EFFECTS OF MICRO-NUTRIENT DEFICIENCIES ON GROWTH AND VITAMIN CONTENT OF THE TOMATO

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Introduction

The nutritive values of food plants and the factors which may modify them are subjects of increasing and timely importance. During the last two summers, extensive experiments have been conducted at this laboratory on the effects of varying concentrations of macro-nutrient elements in the nutrient medium on the vitamin content of tomato fruits and turnip greens (8, 19, 20). A survey of the effects of micro-nutrient deficiencies on the vitamin content and mineral composition of tomatoes is reported in this paper.

Micro-nutrient elements, that is, those mineral elements essential in minute quantities for growth and reproduction of higher plants, have received much attention in recent years. The early research of MAZÉ (31, 32, 33) and others stimulated this interest, and the list of essential elements has been expanded to include manganese (34, 1), boron (49, 53), zinc (47, 49), copper (27, 48), and molybdenum (2, 4) and is probably now incomplete (26).

There have been several reports that various deficiencies of the micro-nutrients may influence vitamin content. POWERS (38) has stated that the addition of boron to soils deficient in this element resulted in a 30 per cent increase in the carotene content of alfalfa. ROY and BAHRT (40) have reported that oranges from zinc-deficient trees contain subnormal amounts of ascorbic acid and that the addition of zinc would restore the ascorbic acid to the normal level. According to HAMDALLAH (18), *Phaseolus multiflorus* or *Zea mays* grown under iron-deficient conditions contains as much or more ascorbic acid than normal plants. COWART (11) has reported that combined applications of copper, manganese, and zinc in sprays to citrus trees may result in greater ascorbic-acid content of the fruits.

Material and methods

Most recent methods of demonstrating deficiency effects of micro-nutrients in solution culture have involved growing plants to various stages of maturity using rigidly purified distilled water, salts free from undesired heavy metals (50, 51), aeration by sintered Pyrex-glass aerators, and Pyrex containers. Reasonable care has been exercised with other factors of the environment which might provide possible sources of contamination. There are two principal difficulties with this method. First, the volume of nutrient supply has been relatively small. It is necessary to change the nutrient medium frequently or to replenish it with ions which have been depleted as a result of plant growth. This is especially true when it is desirable to grow plants to maturity. Second, the aerators have been subject to clogging from algae, dust, or other particles. It is also difficult to se-

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cure uniform aeration in replicated cultures. An apparatus designed especially for nutritional studies in the greenhouse eliminates many of these difficulties (fig. 1).

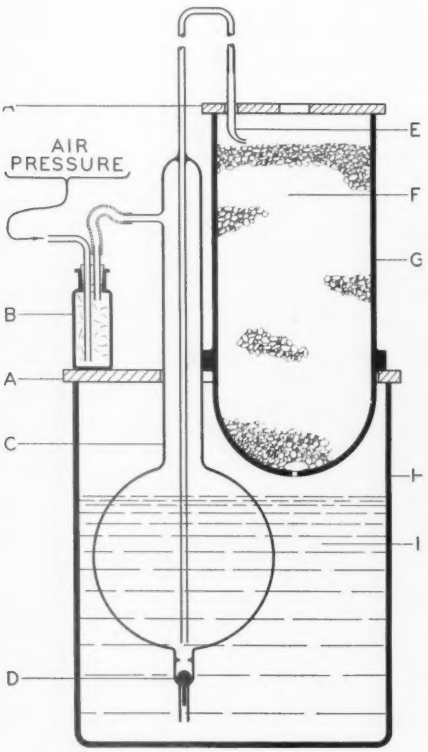

Fig. 1 (*left*)

clude light, and over it a second coat of aluminum paint was applied to prevent excessive heating of the nutrient solution. A strip of black scotch tape (*L*) covered an unpainted area and could be removed when necessary to examine the level of the solution. Three-fourths inch plyboard (*A*) was placed over the top of the jar and painted with three coats of

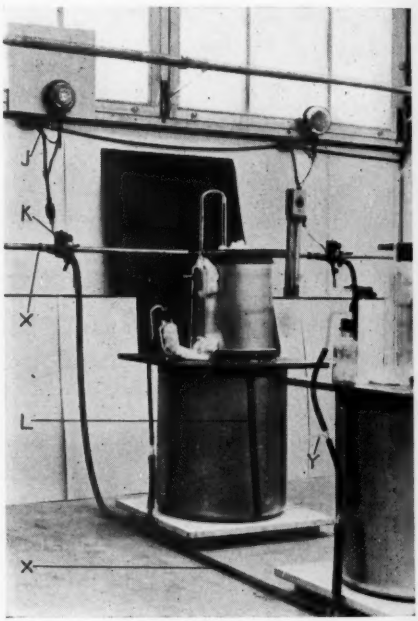

Fig. 1 (*right*)

FIG. 1.—Left, diagram of Pyrex-glass apparatus used to grow tomato plants to maturity using nutrient solutions deficient in micro-nutrient elements. Right, arrangement of apparatus in greenhouse: *A*, $\frac{3}{4}$″ plyboard; *B*, 200-cc. wide-mouthed bottle filled with cotton; *C*, trap; *D*, Pyrex-glass valve; *E*, Pyrex-glass delivery tube; *F*, Pyrex gravel; *G*, round-bottomed Pyrex jar; *H*, Pyrex jar; *I*, nutrient solution; *J*, General Electric time switch 86×843; *K*, electrically controlled solenoid valve; *L*, black scotch tape; *X*, $\frac{3}{8}$″ air line of galvanized iron pipe; *Y*, T-tube with exposed arm made from capillary tubing.

The apparatus consisted of a round, flat-bottomed Pyrex jar (*H*) 16 inches in diameter and 16 inches high, with a capacity of 39 liters of nutrient solution when filled to within 4–5 inches of the top. The jar was first painted on the outside with black enameloid paint to exclude light, and over it a second coat of aluminum paint was applied to prevent excessive heating of the nutrient solution. A strip of black scotch tape (*L*) covered an unpainted area and could be removed when necessary to examine the level of the solution. Three-fourths inch plyboard (*A*) was placed over the top of the jar and painted with three coats of Vinylite plastic paint.[4] Condensation of moisture occurred on the plyboard, and in so far as could be determined the plastic paint was inert and did not contribute any ions to the cultures. The plyboard in

[4] Obtained from Barrett Varnish Company, Cicero, Illinois (No. 5003 clear Vinylite solutions).

turn supported a second round-bottomed Pyrex jar (*G*) with a $\frac{1}{4}$-inch hole at the bottom to permit draining of the nutrient back to the reservoir. These jars were especially constructed with a glass flange approximately 6 inches above the bottom to act as support. They were painted as in (*H*) above. The jars were filled with Pyrex gravel, which consisted of crushed glass from colloidal to walnut sizes. The trap (*C*) was made from 51-mm. outside-diameter Pyrex glass tubing which was sealed to a 5-liter boiling flask. The inside delivery tube was made from 8-mm. Pyrex glass tubing, which was widened at the mouth (*E*) to deliver a sheet of solution at the surface of the Pyrex gravel. A valve (*D*) was sealed in at the bottom of the 5-liter boiling flask.

The seedlings were inserted in the Pyrex gravel substrate (*F*) with the top of the plant protruding through the plyboard (*A*). Cotton was inserted between the stem of the plant and the plyboard and between the plyboard and delivery tube (*E*). Cotton was also inserted in all openings of the plyboard (*A*) which supported the trap (*C*) and jar (*G*). Thus the interior of the apparatus was protected from possible dust and other impurities from the external atmosphere.

A time clock (*J*), operated so that current could be turned on for any desired portion of its complete cycle of 30 minutes, was inserted in the air line (*X*) leading from the compressor to the apparatus. For our purpose, the clock was so regulated that the current was turned on for 15 seconds in each 30-minute cycle. The current opened the solenoid valve (*K*)[5] to admit air pressure, which was filtered to the apparatus through the cotton at (*B*).

[5] Obtained from Minneapolis-Honeywell Regulator Company, Minneapolis, Minnesota (valve No. V437A).

Thus, once every half hour for the duration of the experiment, air pressure was admitted to the apparatus. The valve (*D*) was seated and the nutrient within the trap passed through the delivery tube (*E*) to the surface of the Pyrex gravel. The nutrient, the quantity of which was controlled by regulating the duration of the flushing period, percolated through the gravel and was returned to the reservoir (*H*). When the air pressure was turned off at the end of the flushing interval, the capillary tubes (*Y*) in the air line expelled air until the pressure within the apparatus was equalized with atmospheric pressure. The pressure of the nutrient (*I*) then forced the valve (*D*) open and the trap refilled with the nutrient.

Such an apparatus provides adequate supply of nutrient for large plants and does not require frequent replacement of solutions. Automatic aeration is provided by the frequent percolation of the nutrient through the substrate. The apparatus may be arranged in any desired experimental design, but one entire replication (consisting of a variable number of cultures) must be supplied with a common air line, solenoid valve, and time clock, thus providing all cultures with the same volume of nutrient during a flushing interval. Water was purified in 12-gallon Pyrex-glass carboys by filling them with distilled water and 100 gm. $CaCO_3$, 100 cc. M/1 K_2HPO_4, and 50 cc. M/1 $Ca(NO_3)_2$. A Pyrex-glass tube with a cotton filter was connected to an air line and extended to the bottom of the carboy. Air was bubbled through the mixture at a sufficient rate so that the $CaCO_3$ and the precipitate formed by the addition of $Ca(NO_3)_2$ and K_2HPO_4 were continually agitated throughout the mixture. Continued agitation was necessary for a period of 8 hours, and the adsorbents were allowed to settle for 36 hours.

The supernatant water, free from heavy metals, is siphoned off through Pyrex tubing. The carboy may be refilled with water without removal of the adsorbents. For each subsequent purification it is necessary to add 100 cc. M/1 K_2HPO_4, 50 cc. M/1 $Ca(NO_3)_2$, and 50 gm. $CaCO_3$. All purified water was tested by the dithizone technique. It was possible to purify 250 gallons in 24 carboys during each 72-hour period.

For the control plants, 39 liters of nutrient solution were used in each culture. The following quantities of nutrients were present in addition to a small quantity of those contributed by the purification process:

Ca	12 m.e./l.
Mg	9
K	9
NH_4	2
SO_4	9
HPO_4	11
NO_3	12
B (as H_3BO_3)	0.5 p.p.m.
Mn (as $MnCl_2 \cdot 4H_2O$)	0.5
Zn (as $ZnSO_4 \cdot 7H_2O$)	0.05
Cu (as $CuSO_4 \cdot 5H_2O$)	0.02
Mo (as 85% MoO_3)	0.05
Fe (as $FeCl_3$)	5.00

For the macro-nutrients it is necessary to purify only four salts, which were prepared as molar solutions, with appropriate dilutions when the nutrient medium was made up. The salts, together with their final molar concentrations, are as follows: $Ca(NO_3)_2$, 0.006 M; KH_2PO_4, 0.0045M; $MgSO_4$, 0.0045 M; $(NH_4)_2HPO_4$, 0.001 M. Methods developed by STEINBERG (**50**) and modified by STOUT and ARNON (**51**) were used for the purification of all salts containing macro-nutrient ions from heavy metals.

Since iron was the only micro-nutrient added in appreciable amounts to the nutrient medium, and since iron salts commonly contain varying quantities of copper and other elements, $FeCl_3$ was the only salt of a micro-nutrient which was purified. The procedure involved a repeated ether extraction of the iron from a hydrochloric-acid solution (**25**). If desirable, ferric chloride may also be separated from molybdenum by the basic acetate method (**28**).

The nutrient solution of any micro-nutrient deficient culture was prepared by adding to the basal nutrient of macro-elements all supplements of micro-nutrient elements, with the exception of the element under consideration. Water was added twice weekly to maintain a constant volume. At the same time the pH was adjusted to 5.5 with HNO_3 (**14**). During the last month of the experiment, when the plants were large, the nutrient was made up to volume with purified water three or four times weekly. When needed, additions of iron were made to the culture solutions.

Chemical methods were used for the determination of ascorbic acid, with a modification described by MORELL (**36**). The aliquot, however, was titrated to an end point with standardized dye. The method of provitamin-A determination is described by ELLIS *et al.* (**16**). It consists of an extraction of diacetone and acetone-soluble pigments, which are transferred to petroleum ether. A separation of the pigments is made on a Tswett column consisting of a mixture of magnesium oxide and Supercell. The top band of pigments (largely lycopene) is discarded and the lower band (largely carotene) is eluted with petroleum ether containing 4 per cent ethanol. The concentration of the pigment is determined in a photoelectric colorimeter previously calibrated with β-carotene. The method is somewhat empirical, but it determines a group of carotenoid pigments consisting mostly of β-carotene. Unpublished data

show good agreement with a rat bioassay on a sample of canned tomato puree. The method is rapid, permitting completion of the initial stages of the extraction and transfer of the pigments to petroleum ether within an hour after the fresh fruits are sampled. Riboflavin[6] was determined by SNELL and STRONG's microbiological method (46).

Mineral determinations were made by the following methods: zinc, COWLING and MILLER (12); iron, SAYWELL and CUNNINGHAM (43); copper, COULSON (10) with modifications suggested by DRABKIN (13); molybdenum, MARMOY (30) with modifications suggested by ROGERS (39); boron, NAFTEL (37); and manganese according to the Official Method (5). The analyses were made on unwashed leaflets, since in many instances the leaflets were dead.

Experimentation and results

On April 20, 1942, inbred seeds of Bonny Best tomatoes were planted in large flats with specially prepared acid-washed sand and watered with purified water adjusted to a pH of 5.5 with HNO_3. This water contained only small quantities of ions contributed by those salts used in the purification process ($Ca[NO_3]_2$, K_2HPO_4, and $CaCO_3$). The seedlings were grown under these conditions until May 31, when they were approximately 3 inches tall, very chlorotic, and purple on the under side of each leaf. Deficiency symptoms of several macro-nutrients were clearly evident. By chemical analyses the seedlings were shown to be deficient with respect to micro-nutrients as well. The cotyledons were removed, uniform seedlings transplanted into the apparatus, and immediately supplied at ½-hour intervals with their respective nutrient solution. The plants became normal in appearance within a period of 1 week, with a healthy green color in leaves and stems. As they developed, the plants were supported by cords suspended from the greenhouse roof, and all axillary growth was removed twice weekly. When all plants were harvested, October 1, the number of internodes was counted on each plant and the aerial vegetative growth divided into three parts, with an equal number of elongated internodes in each part. The expanded leaflets from each of these divisions were preserved for micro-nutrient analyses.

During the course of the experiment, each fruit was picked on the morning of the day that complete color change had occurred. On each plant the second, fourth, and sixth fruits to ripen were allocated for chemical analyses, while the first, third, fifth, and seventh fruits were used for vitamin determinations. When picked, the fruits for vitamin assays were immediately weighed and transverse sections weighing approximately 10 gm. taken from the equatorial portion.

Forty-nine cultures were used in a Latin square (17), with seven treatments and seven replications randomized by the use of TIPPETT's tables (52). All data were reduced and analyzed by means of the analysis of variance (45), and the t test was used for determining significant differences. Odds as great or greater than 99:1 were accepted as proof that the observed differences were not due to the errors of random sampling. The nature of the results was such that it was impossible to maintain orthogonality. In such cases, only actual data were used in the analysis of variance, and a second analysis was computed using substituted values obtained by YATES's method (54), with one degree of freedom deducted for each substituted value. In no case did

[6] The aid of Miss R. MACHATA in preparing and incubating bacterial cultures is appreciated.

the latter procedure change the conclusions that could be drawn by the former method.

Analyses of variance were computed for each character, and the results are compiled in table 1. It is evident that significant differences between treatments are inherent in the data for vegetative growth and fruit production, since all F values are well in excess of those required for a P value of 0.01. It is also evident that the effects of environment when measured by replication did not produce statistically significant differences in any of the characters used as criteria of growth and fruitfulness, except the dry weight of the vine. The differences in the environment measured in this experiment may be due to differences between replications resulting from minute variations in the amount of nutrient delivered during the flushing period or to differences in the position of the plants in the greenhouse. The latter possibility seems the more probable, and in this case the difference would result from variations in climatic environmental factors. In this connection, the data for fresh weight of vine showed no significant effects resulting from position in the greenhouse.

TABLE 1

RESULTS OF ANALYSES OF VARIANCE ON CHARACTERS FOR WHICH DATA WERE RECORDED

CHARACTERS	F VALUE	
	Between treatments	Between replications
Vegetative growth*		
Height of plants	26.45	0.57
No. of internodes	16.47	0.81
Fresh weight of vine	114.87	2.27
Dry weight of vine	63.58	4.08
Fruit production*		
Total fresh weight of fruit per plant	28.15	1.84
No. of fruit ripened per plant	19.66	1.34
No. of immature fruit per plant	24.75	2.27
Total no. of fruit per plant	37.68	1.32
Vitamin content of fruit†		
Ascorbic acid	8.13	1.77
Provitamin A	1.73	1.72
Riboflavin	3.64	11.80

*F values 3.47–3.56 required for P value of 0.01 for both statistics.

†F values 2.97–2.99 required for P value of 0.01 for both statistics.

It is evident that ascorbic-acid and riboflavin content were significantly influenced by treatment, while no significant difference between treatments was demonstrable with respect to provitamin A. Riboflavin was the only vitamin tested which was significantly influenced by position in the greenhouse. The possibility of the effects of variations in technique being confounded with the effects of plant position is not indicated with the number of analyses used for each vitamin (approximately 125).

Since significant effects with respect to treatment are shown in almost all the characters studied, data for vegetative growth and fruit production are given in table 2. The control plants were tall, thick-stemmed, and had healthy apical growing points. The leaves were dark green, fully expanded, and had a few extra leaflets which developed from buds at the junction of the leaflet and petiole. The fruit production of these plants was comparable in quantity with that produced by plants under optimum treatments of previous experiments (**19**), although mature fruit size was somewhat reduced. The fruits were firm and had normal colorations during ripening. Both leaflets and fruits contained normal quantities of micro-nutrients (**7**). During the early part of September, when the plants were large and had produced six mature fruits, the laminae of the lowermost leaves became chlorotic, a condition not curable by additions of iron, and there was some purple spotting. The chlorosis and purpling did not progress upward on the plants (figs. 2*F*, 3*A*). This condition sometimes occurs in old field-grown plants.

Manganese deficiency

The first symptom of manganese deficiency was a spotted chlorotic condition which appeared when the plants were 10 weeks old (4 weeks after transplanting). The leaflets at this time were not so large as the control leaflets, although the plants were growing vigorously. The chlorosis was first observable in the blades of the leaflets near the top of the plant but soon became general and was accompanied by a yellowing of the entire leaflet lamina. The leaflets became completely brown and subsequently developed necrosis at the tip or edges, or in some instances at the midvein of the leaflet (fig. 3*B*). When the plants were 19 weeks old, leaflets of the entire upper three-fourths of these plants appeared dead (fig. 2*C*). The stem and petioles began to develop intermittent brown spotting, and the top part of the stems began to turn yellow. At the conclusion of the experiment the top part of the stem was entirely brown and dried up. The final height of the manganese-deficient vines, as well as the fresh and dry weights, was significantly less than that of the control plants (table 2).

Fruit production was also significantly less in all characters used as criteria of fruitfulness, except for the mean weight of ripe fruits where statistically significant differences were not demonstrable. This deficiency was also evidenced by fruit symptoms. In normal ripening, the stem end of the fruits is the last portion to become completely red. This is also true of the manganese-deficient fruits, but the fruits were somewhat more yellow than normal ones during all stages of development until ripening occurred. At the stem-end portion, yellow-green areas persisted during ripening and never became red. In these green areas pin points of brown occurred at random. The brown spots may occur prior to the time that the fruits start to become red, although more commonly they occurred subsequent to the onset of ripening. One manganese-deficient fruit was allowed to remain on the vine until it had completely dried up. The green areas remained clearly evident through the soft-ripe stage and during the drying process.

Replicates of the manganese-deficient treatment showed to varying degrees the symptoms just mentioned. Chemical an-

TABLE 2

GROWTH MEASUREMENTS AND FRUIT PRODUCTION, GIVING TREATMENT MEANS TOGETHER WITH THEIR STANDARD ERRORS

Characters	Control plants	Deficient treatment					
		Mn	Zn	Mo	Cu	B	Fe
Vegetative growth							
Height of plants (cm.)	307± 5.6	179±12.6	239 ± 18.4	299± 0.4	163±10.7	291± 13.1	172± 14.5
No. of internodes	44± 1.2	29± 1.3	36± 2.9	42± 1.8	28± 1.0	42± 1.1	32± 0.8
Fresh weight of vine (gm.)	2293± 48.5	685±27.1	1075±195.9	2332±135.5	213±45.0	2350± 69.5	608±130.0
Dry weight of vine (gm.)	339± 22.3	99± 5.3	179± 41.5	353± 28.6	28± 5.2	380± 20.3	78± 19.6
Fruit production							
Total fresh weight (gm.) of fruit per plant	1747±228.4	447±84.8	760± 73.9	1925±299.7	72±44.2	2393±130.7	401±113.2
No. of fruit ripened per plant	15± 1.7	6± 1.0	11± 1.5	20± 2.8	2± 1.0	21± 1.7	4± 0.7
No. of immature fruit per plant	21± 2.9	2± 0.6	1± 1.2	22± 3.6	0± 0.0	25± 3.2	6± 3.7
Total no. of fruit per plant	36± 4.0	8± 1.2	12± 0.8	42± 4.7	2± 1.0	46± 3.5	10± 4.0
Mean weight ripe fruit (gm.)	92± 5.0	81± 7.7	68± 6.0	77± 3.9	50± 8.6	87± 8.6	84± 5.1

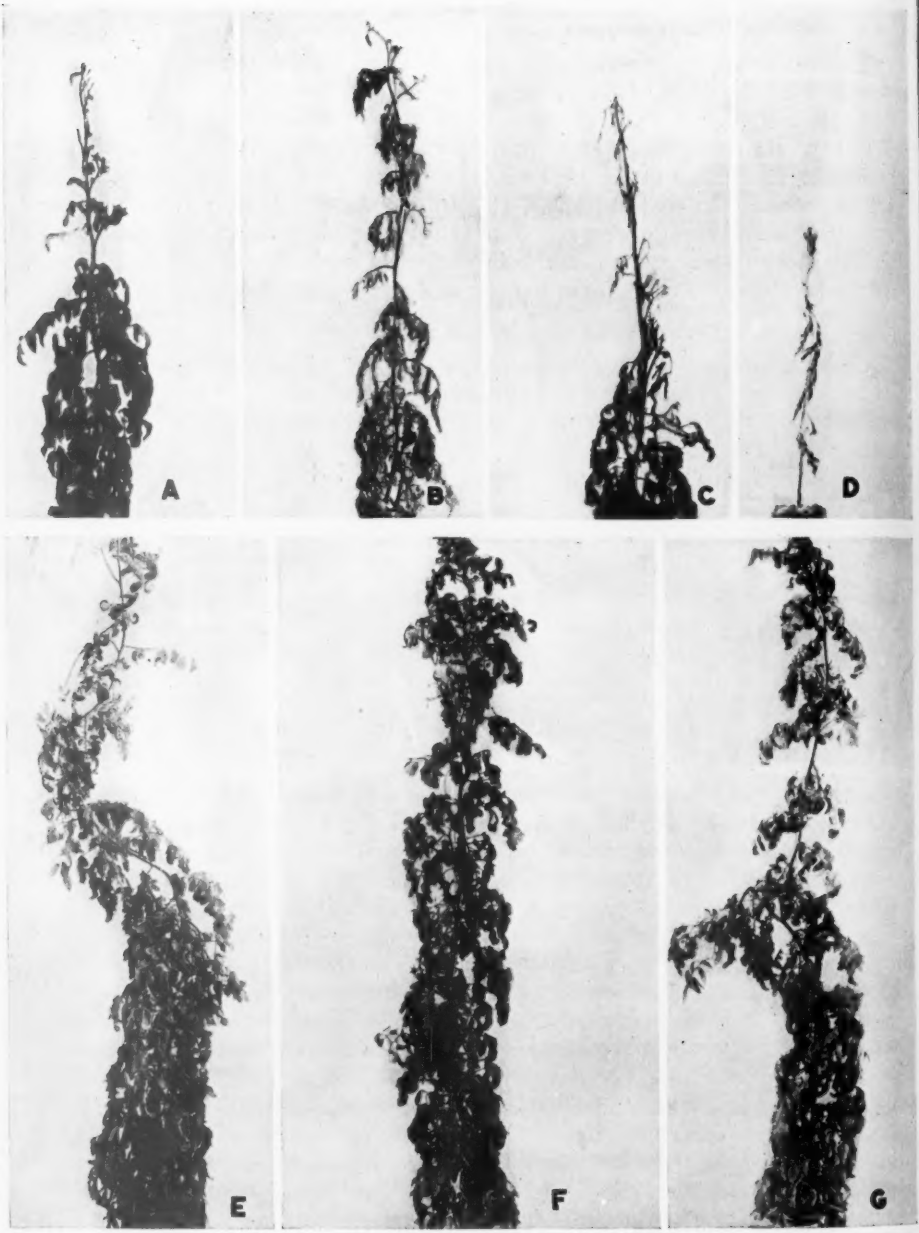

FIG. 2.—Typical tomato plants grown with various micro-nutrient deficient culture solutions together with control plant: *A*, iron-deficient; *B*, zinc-deficient; *C*, manganese-deficient; *D*, copper-deficient; and *F*, control plant. *E*, plant grown with nutrient with no added molybdenum, and *G*, with no added boron, in nutrient medium.

alyses on the second, fourth, and sixth fruits and the leaflets from deficient plants provide an index of the severity of the deficiency (table 3). When compared with other analytical data, these show that the control fruits had normal amounts of manganese (6). The lower leaflets of the control plants contained approximately 5.5 times as much manganese per unit dry weight as the leaflets near the top of the plant. Thus there is a gradient of manganese content within the plant which increases from top to bottom. The data for the manganese content of fruits are computed on a fresh-weight basis. Since most tomato fruits contain 7–8 per cent dry matter, the value given in the table may be multiplied by an approximate factor of 12 to obtain values comparable with those of the leaflets. It is evident that the concentration of manganese in fruits is less on a dry-weight basis than in leaflets from any portion of the plant. The deficient fruits contained about one-ninth as much manganese as did those produced on the control plants. The leaflets of the deficient plants contained only small amounts of manganese, and there were no

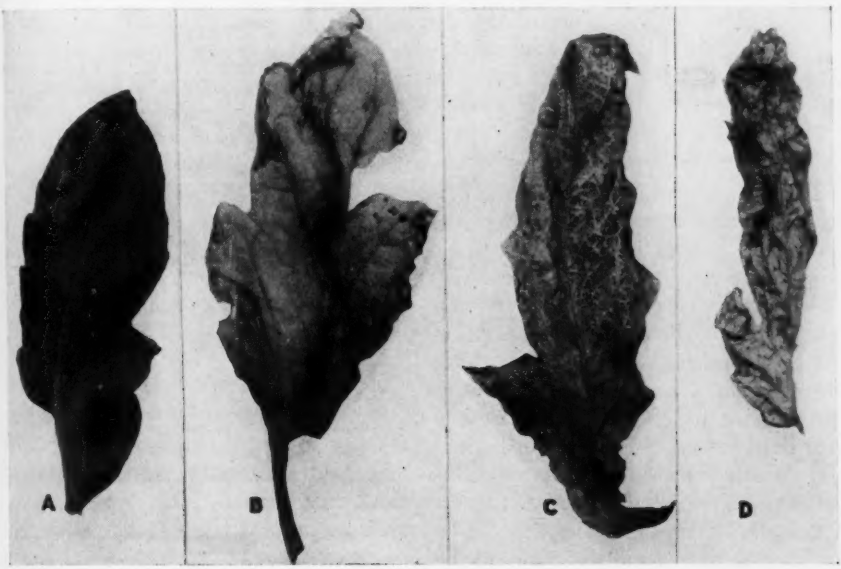

FIG. 3.—Typical leaflets from tomato plants grown with various micro-nutrient deficient culture solutions together with control: *A*, control; *B*, manganese-deficient; *C*, zinc-deficient; and *D*, copper-deficient.

TABLE 3

MANGANESE ANALYSES (P.P.M.) OF PLANTS GROWN IN COMPLETE AND MANGANESE-DEFICIENT CULTURE SOLUTIONS

PLANT PART	COMPLETE		MN DEFICIENT	
	Mean ± S.E.	No. of analyses	Mean ± S.E.	No. of analyses
Fruit*	1.7 ± 0.17	11	0.2 ± 0.02	9
Leaflets†				
Top one-third of plant	69.7 ± 9.08	7	5.6 ± 1.33‡	5
Middle one-third of plant	248.3 ± 29.95	7	5.3 ± 0.94	6
Lower one-third of plant	398.0 ± 23.79	7	5.4 ± 0.67	7

* Computed per unit fresh weight of fruit.
† Computed per unit dry weight of leaflets.
‡ Many leaflets dead at time of harvest.

significant differences in the concentration of this element in leaflets from different positions on the plant. The gradient found in controls was not evident in the deficient plants. The relative proportion of manganese in fruits as compared with leaflets was greater in the manganese-deficient plants than in the controls, indicating some accumulation of manganese in the fruit at the expense of vegetative parts when the supply of this element is limited.

Zinc deficiency

The symptoms of zinc deficiency appeared rather late in the life-span of the plant. The first evident symptom was a chlorosis between the veins of the lowermost leaflets. The veins and veinlets remained green. The chlorotic condition progressed upward on the plant, and, when the leaflets on the middle part were affected, the lowermost leaflets were a clear light brown which was not limited to the blades but included veins and veinlets as well. When the leaflets at the top of the plant were chlorotic, the middle leaflets were brown and the blades of the lowermost leaflets were speckled (fig. 3*C*). These stages followed in sequence, and in some cases the upper leaflets appeared normal in all respects while the lowermost were completely brown and speckled. Eventually, necrotic areas appeared at the margins of the leaflet and progressed toward the middle. At the conclusion of the experiment, all leaflets on a plant were a clear brown in color, speckled, and had necrotic areas (fig. 2*B*). The leaves as a whole were usually smaller than those of control plants, and there was a tendency for the leaflets to curl inward.

Vegetative growth was significantly less than that of control plants (table 2). Fruit production was also significantly less. As was the case for manganese, certain characteristic symptoms were evident on zinc-deficient fruits. They were small as compared with the controls, and the coloring of the fruit was a mottled red or a reticulum of red and yellow-orange. The mottling was particularly prevalent near the distal portion and, as the fruit matured, progressed toward the base. It was not evident on all fruits, although it was most prevalent on those from plants which exhibited the more severe deficiency symptoms.

TABLE 4

ZINC ANALYSES (P.P.M.) OF PLANTS GROWN IN COMPLETE AND ZINC-DEFICIENT CULTURE SOLUTIONS

Plant part	Complete		Zn deficient	
	Mean ± S.E.	No. of analyses	Mean ± S.E.	No. of analyses
Fruit*	1.9 ± 0.13	9	0.6 ± 0.06	8
Leaflets†				
Top one-third of plant	28.0 ± 5.50	5	23.4 ± 5.84	5
Middle one-third of plant	26.9 ± 3.43	5	14.4 ± 1.61	5
Lower one-third of plant	29.8 ± 3.36	5	16.5 ± 1.92	5

* Computed per unit fresh weight of fruit.
† Computed per unit dry weight of leaflets.

Analytical data (table 4) indicate a uniform distribution of zinc among leaflets of control plants and that the concentration found in control fruits is approximately 80 per cent of that of the leaflets. The content of zinc in the leaflets of deficient plants compared with that of the controls shows that, while no significant differences occur in the upper one-third of the plant, significantly less zinc is present in the lower two-thirds. Relatively large standard errors are involved in the analytical data of the top leaflets, and greater variability with respect to zinc content was evident between plants in both the control and the deficient treatment. Fruits produced by

zinc-deficient plants contained approximately 30 per cent as much zinc as those from controls and approximately 30 per cent of the amount in deficient leaflets.

Iron deficiency

When the seedlings were transplanted into cultures which contained no added iron, they were about 3 inches tall and extremely chlorotic. They were deficient in iron as well as in other elements. Although they became normal in appearance, they failed to make appreciable growth after transplanting, and it was apparent that some addition of iron was necessary if the plants were to produce fruits. Since the primary objective of this experiment was to produce iron-deficient fruits for vitamin analyses, and since the distribution of iron in fruit and vegetative parts of the plant was unknown, it was inadvisable to add iron directly to the nutrient medium, as fruits containing relatively large quantities of iron might result. Statements in the literature imply that iron is relatively immobile in plants (**35**). If true, it should be possible theoretically to spray the lowermost leaves of a plant with a stable emulsion of iron so that they would become green. If this occurred, the manufactured foods might then be conducted to fruits on the upper portions lacking the iron. Accordingly, an emulsion of ferric citrate (concentration of 1:1000) was prepared (**24**) and atomized on to the lower leaflets July 1, 1942. Within a period of 2 weeks, not only the lower leaflets but the unsprayed upper leaflets had become green on five of the seven plants. In two cases the application of the emulsion resulted in no change, either in upper or lower leaflets, and the plants were sprayed again on July 20. Subsequently all leaflets of these two plants also became green. The five plants which had become entirely green were maintained in their respective cultures with no further addition of iron, and ultimately—after a period of about 6 weeks—three of the plants again began to develop characteristic iron-deficiency symptoms. In two of these cases the symptoms were so severe that the young expanded leaflets at the apex were a yellowish white color. At the conclusion of the experiment the three plants were light green in color, with a definite yellowish white color at the top of the plant (fig. 2*A*). The fruits and vegetative parts contained significantly less iron than did the controls. The remaining four plants (including the two which were sprayed twice) failed to develop striking deficiency symptoms and did not contain appreciably less iron. In subsequent data, only the results of the three clearly deficient plants were included.

Vegetative growth was clearly affected by the limited supply of iron (table 2; fig. 2*A*). In all characters used as criteria of vegetative vigor, significant differences are demonstrable between the data for iron-deficient and for control plants. It is also evident that the treatment seriously affected all characters used as criteria of fruitfulness, except the size of mature fruit, for which no statistically significant differences are evident. The deficient fruits which developed were a waxy, yellowish green color and though mature remained green longer than did control fruits. The ripening process with its accompanying red color was delayed; the fruits finally became entirely pink, but a dark red color was never attained.

From table 5 it is evident that the top leaflets of control plants contained less iron than those located at the bottom of the plant, since a mean difference of 54 ± 18.4 p.p.m. (10 degrees of freedom are available, $t = 2.93$) is demonstrable.

The control fruits contained approximately 20 per cent as much iron as the leaflets on a dry-weight basis. Fruits produced by the three deficient plants contained significantly less iron than control fruits, since a mean difference of 1.9 ± 0.53 p.p.m. (9 degrees of freedom are available, $t = 3.58$) is evident with respect to iron content. The leaflets of deficient plants also contained significantly less iron than the controls, and the same distribution of iron was maintained in the plant when the supply of iron was limited.

TABLE 5

IRON ANALYSES (P.P.M.) OF PLANTS GROWN IN COMPLETE AND IRON-DEFICIENT CULTURE SOLUTIONS

PLANT PART	COMPLETE		FE DEFICIENT	
	Mean ± S.E.	No. of analyses	Mean ± S.E.	No. of analyses
Fruit*	3.3 ± 0.41	6	1.4 ± 0.33	5
Leaflets†				
Top one-third of plant	175 ± 14.9	6	74 ± 18.4	3
Middle one-third of plant	199 ± 9.0	6	128 ± 8.7	3
Lower one-third of plant	229 ± 10.8	6	167 ± 8.6	3

* Computed per unit fresh weight of fruit.
† Computed per unit dry weight of leaflets.

COPPER DEFICIENCY

With the exception of the iron-deficient plants, those deficient in copper were the first to show recognizable symptoms. A gray-green coloration of the entire plant was first discernible about June 25, when the plants were 67 days old (25 days after transplanting), and was clearly evident by June 30. This coloration was followed approximately 1 week later by distinct chlorosis of the lower leaflets. The latter condition first appeared immediately adjacent to the veins and gradually spread into the islets. The veins became dark brown and apparently filled with a brownish deposit. Microscopic examination showed no cellular deterioration at this stage. Subsequently the chlorotic lower leaflets became bronzed, extremely flexible, and the leaf petioles formed unusual angles with the stems. The bronzed condition was followed by complete browning of the leaflets, with necroses at the margins and black veins (fig. 3*D*). Lastly a clear brown color with an accompanying brittle quality of the leaves indicated death. Brown woody spots developed on the surface of the stems and petioles. The stems were thin and spindly, while all leaves and leaflets were relatively small. By September 2, when the plants were 135 days old, five of the seven replications had only three or four green leaves at the apex of the plant. At the conclusion of the experiment, when the plants were 165 days old, most of the stem and all leaves were dead on each plant (fig. 2*D*).

From table 2 it is clear that a lack of copper resulted in the smallest plants in the experiment. Highly significant differences are demonstrable between the data for copper-deficient and the control treatments in all characters used as criteria of vegetative vigor. Similarly, the data for fruitfulness show highly significant differences for all characters. Each plant flowered and set two fruits early in the experiment, and all subsequent flowers abscised at the pedicel joint. The mature fruits were small (table 2), but the ripening process with respect to colors which developed and rate of maturation appeared comparable with that of the control fruits.

The copper content showed no consistent relationship to symptoms which developed on the deficient plants. Furthermore, the variability of the results could not be explained or correlated with

the growth data, hence the copper analyses are not reported. It is planned to obtain further analytical results from copper-deficient and normal tomato plants in subsequent experimentation.

Molybdenum and boron

The data for the growth of plants grown with nutrient solutions to which no molybdenum was added (table 2) agree within the limits of error with similar data for control plants, and in no case is a significant difference demonstrable. There was no evident external vegetative symptom which could be correlated with a limited supply of this element (fig. 2*E*). The data for the number of ripe and developing fruits, the total number of fruits, and the total fresh weight of fruit produced agree closely with similar data for the control plants; but there are indications that the size of ripe fruits produced by molybdenum-deficient plants was smaller than that of control fruits. A mean difference of 15 ± 6.3 gm. (33 degrees of freedom are available, $t = 2.38$) is shown in the data, and while this difference is not significant according to the arbitrary standards required in this experiment, fruit size was the only external character examined which showed indication of less growth as a result of limited molybdenum supply.

The results of molybdenum analyses are given in table 6. It is evident that lower leaflets of control plants contained significantly more molybdenum than top leaves, since a significant mean difference of 2.29 ± 0.745 p.p.m. is demonstrable in the data (12 degrees of freedom are available, $t = 3.04$). The concentration of molybdenum in the fruits on a dry-weight basis was approximately 30 per cent of that of leaflets. The fruits contained slightly less than 0.01 ± 0.001 p.p.m. of molybdenum, and its concentration in the leaflets of the treated plant was approximately 10 per cent of that of the controls. In these plants there does not seem to be any uniform trend for molybdenum content of the leaflets, although there is an indication that lower leaflets contain more than those of the middle portion of the plant. The mean difference

TABLE 6

Molybdenum analyses (p.p.m.) of plants grown in complete and molybdenum-deficient culture solutions

Plant part	Complete		Limited Mo supply	
	Mean ± S.E.	No. of analyses	Mean ± S.E.	No. of analyses
Fruit*	0.06 ± 0.005	6	0.01 ± 0.001	11
Leaflets†				
Top one-third of plant	2.53 ± 0.554	7	0.26 ± 0.059	7
Middle one-third of plant	3.48 ± 0.365	7	0.21 ± 0.034	7
Lower one-third of plant	4.82 ± 0.512	7	0.39 ± 0.059	7

* Computed per unit fresh weight of fruit.
† Computed per unit dry weight of leaflets.

of 0.18 ± 0.068 p.p.m. ($t = 2.65$) is not significant.

Boron was included in the nutrient solution supplied to the control plants to preclude possibility of a boron deficiency. The treatment receiving no boron was included largely as a test of the boron-supplying capacity of Pyrex glass. No significant differences with respect to any character used as a criterion of growth and fruitfulness were obtained between the control plants (receiving added boron) and those receiving nutrient to which no boron was added (table 2; fig. 2*G*). Boron analyses of leaflets from plants of both of these treatments were made when the plants were 54 days old (14 days after transplanting) and again when 135 days old. At the earlier date the leaflets of plants in the first treatment contained 62 p.p.m. of boron, while leaflets from the

latter treatment contained 39 p.p.m. of boron. When the plants were 135 days old the boron content of leaflets taken from plants of both treatments was the same. Thus in this experiment the Pyrex-glass gravel supplied adequate boron for normal growth and reproduction.

VITAMIN CONTENT OF FRUITS

The first, third, fifth, and seventh fruits which ripened on each plant were assayed for ascorbic acid, riboflavin, and provitamin A. No attempt was made to select fruits from any particular cluster, and the usual situation with respect to the control plants was that the first and third fruits ripened on the first fruit cluster, the fifth fruit was harvested from the second fruit cluster, and the seventh fruit ripened on the third fruit cluster. One additional point is pertinent to any interpretation of the results. The first fruits obtained from each of the seven replications in any one treatment ripened within 6 days of one another, and a similar situation was found for the third fruits. The fifth fruits ripened within 10 days and the seventh fruits within 2 weeks of one another. While the extremes with respect to ripening date overlap, there are clearly different mean dates for the time at which the first, third, fifth, and seventh fruits ripened. Furthermore, the different treatments ripened their first fruits at approximately the same time (within a week of one another), so that the first fruit from any replication of any deficient treatment ripened under environmental conditions similar to those of the first fruit from any replication of the control treatment. Any differences within or between treatments with respect to the ripening date of any given fruit appear to be distributed at random in the data.

Since the results previously discussed have characterized the deficiency conditions produced in this experiment, and since mineral analyses were made on both fruits and leaflets of each plant, it was possible in most cases to determine which plants included in any treatment were clearly deficient in any given mineral element and whether or not they produced fruit deficient in a given element. It is evident from the data that four plants of the treatment where the supply of iron was limited did not develop iron-deficiency symptoms, and the results obtained from iron analyses of the fruits from these plants were comparable with those of controls. The vitamin data on these fruits were discarded. Using similar criteria, the results from two plants from the treatment which had no zinc added to the nutrient medium were not used. The results of vitamin analyses on fruits from all other plants were considered valid for purposes of this experiment.

Significant differences between treatments are inherent in the data for ascorbic acid and riboflavin, while none were demonstrable for provitamin A (table 1). Riboflavin was the only vitamin in this experiment which was significantly affected by position of the plants in the greenhouse. One other point illustrated in the analysis of variance is also pertinent to any interpretation of the results. The data for each vitamin were tested for the possible presence of significant differences between values for the first, third, fifth, and seventh fruits. This is in effect a further test of the influence of environment, since such a classification is chronological and the last fruit to ripen on a plant matures under environmental conditions different from those of the first fruit. In this respect, significant differ-

ences between fruit sequences were found for riboflavin only. Thus the riboflavin content of tomato fruits seems to be greatly affected by environmental conditions, since (*a*) significant differences with respect to riboflavin content were correlated with the time at which the fruits were harvested and (*b*) riboflavin content was also correlated with the position of the plant in the greenhouse. This in no way invalidates inter-treatment comparisons, since the effects of the position of the plant and the time at which a fruit ripened were measured and removed from inter-treatment comparisons in the analysis of variance.

TABLE 7

VITAMIN ANALYSES OF TOMATOES GROWN WITH MICRO-NUTRIENT DEFICIENCIES

Treatment	Ascorbic acid*		Riboflavin†		Provitamin A‡	
	Mean±S.E.	No. of analyses	Mean±S.E.	No. of analyses	Mean±S.E.	No. of analyses
Complete nutrient	20.0±0.46	26	0.30±0.010	23	4.0±0.22	24
Mn deficiency	20.2±0.49	18	0.33±0.010	19	4.1±0.20	17
Zn deficiency	21.5±0.70	17	0.26±0.017	18	4.4±0.37	14
Cu deficiency	20.5±1.05	6	0.33±0.013	5	5.1±0.38	6
Mo supply limited	19.6±0.39	27	0.31±0.013	26	4.0±0.19	26
Fe deficiency	26.1±0.55	6	0.34±0.018	5	3.4±0.30	6
B not added to nutrient	19.6±0.54	26	0.30±0.008	26	4.1±0.28	26

* Milligrams per 100 gm. fresh weight fruit.
† Micrograms per gm. fresh weight fruit. ‡ Micrograms per gm. fresh weight fruit.

The results of the vitamin analyses are given in table 7. Individual errors which measure variation between plants and between fruits within plants were computed for each treatment in these data. Significantly more ascorbic acid was found in fruits when the supply of iron was limited than in control fruits, since a significant mean difference of 6.1±0.72 mg. per 100 gm. is shown in the data (30 degrees of freedom are available, $t=8.47$). The ascorbic-acid values for fruits produced in other treatments did not differ from those of the control treatment within the limits of error in the experiment. When data for the deficient treatments are compared individually with those of the control treatment, no significant difference in the riboflavin content of the fruits is demonstrable. It is evident that the individual errors are high, owing to the fact that they include variability obtained as a result of positional effects in the greenhouse and the effects of sequence number. It is possible, by using a generalized error from the analysis of variance (where some sources of variability other than those inherent within treatments are measured and removed), to demonstrate significant differences between treatments. Regardless of the presence or lack of significance, however, the differences obtained are small and probably of no practical value.

The data obtained with respect to provitamin A indicate that the treatments did not influence the content of this vitamin in the fruits. The variability is high, and the analysis of variance would indicate that it occurs at random, since in this experiment no significant effects of position in the greenhouse or of

the time of fruit ripening are demonstrable in the data. The values reported for provitamin-A content of these fruits are in agreement with those of previous experiments (15).

Discussion

The evidence necessary to establish the essentiality of mineral elements for the growth of higher plants has been adequately discussed (3, 26). A quantitative approach as a method of physiological research is necessary in order to establish the essential nature of many elements which may be required in amounts smaller than the incidental impurities which can be removed from culture solutions by present techniques. It is also essential to establish the degree or the severity of the deficiencies, which may be correlated with other variables. It seems obvious that the terms somewhat deficient, deficient, or very deficient are relative in meaning. Their use and interpretation, however, can be more exact if they are defined in terms of chemical analysis, which provides a scale of reference. This procedure in no way invalidates or discards the use of vegetative symptoms as a criterion.

In this experiment the development of external deficiency symptoms on vegetative parts of the plant can be closely correlated with data obtained by chemical analyses. Those plants developing symptoms which could be correlated with a limited supply of an element were found upon analysis to contain significantly smaller amounts of that element than control plants. Conversely, plants which failed to develop deficiency symptoms were found to contain quantities of a given element comparable with that found in controls. The treatment involving no addition of molybdenum to the nutrient medium would be an exception to the latter statement. Within a treatment, the replications differed in the severity of the symptoms which they developed, and plants which developed the most severe symptoms invariably contained the smallest quantities of the element under consideration.

Since ARNON and STOUT (4) have described external molybdenum-deficiency symptoms on the tomato plant, we have avoided designating those plants which were grown with a limited molybdenum supply as deficient. In this experiment no external deficiency symptoms were recognizable, but chemical analyses have shown that they contain approximately one-tenth of the quantity of molybdenum present in control plants. As far as we are aware, no published data are available concerning the molybdenum content of plants with clearly developed external deficiency symptoms. Such data would be helpful in an interpretation of these results. It is possible that contaminants present in these cultures supplied enough molybdenum to support growth and fruitfulness comparable with that obtained in the complete nutrient. If so, certainly only very minute quantities of molybdenum are necessary for normal growth of the tomato plant.

Mineral analyses on leaflets from different parts of the plant have indicated correlations involving the distribution of some micro-nutrients within the plant. There was a suggestion that the first external symptom of a deficiency condition occurred in that part of the plant where the content of a micro-nutrient was small as compared with other parts. Also, at any given time, the severity of the external symptoms seemed to be greatest in that part of the plant. For instance, lower leaflets of all control plants contained

significantly more iron and manganese than did leaflets at the top of the plant. In iron- and manganese-deficient plants the first symptoms of the deficiency appeared at the top of the plant and subsequently became more severe at that point.

Recently the concept that manganese is concerned in the elaboration of ascorbic acid has received some support. This concept was first developed from biochemical studies of liver tissue (**41, 42**), although recent experiments (**9**) have failed to confirm this conclusion. HESTER (**21–23**) has stated that the addition of manganese to soils deficient in this element resulted in increased content of ascorbic acid in tomato fruits. The results reported here have shown that the ascorbic-acid content of manganese-deficient tomato fruits is not different from that of control plants. The deficient condition produced in this experiment would not be tolerated in field-crop production, owing to the limited growth and fruitfulness. This seems to indicate that manganese is not directly concerned in ascorbic-acid synthesis but in no way eliminates the possibility that additions of manganese to soils may alter their physical and chemical properties. In such instances the effect of manganese would thus be indirect. This is discussed elsewhere (**29**).

In this experiment a relatively large difference in the content of manganese, zinc, iron, molybdenum, and probably copper in the plant was experimentally induced. The micro-nutrient content of these plants is in some cases lower than any values previously reported. It may be deduced that a limited supply of zinc, copper, manganese, and molybdenum showed no correlation with the vitamin content of tomato fruits under these conditions. The results indicate the necessity of examining with care data in which significant correlations exist, since other conditions in experimental technique may be of primary importance. In this respect it should be evident that interpretation of these results depends on the following interpretations of a deficient condition. If the deficient condition results from lack of an adequate quantity of an element necessary for some phase of cellular metabolism, an understanding of the role of various micro-nutrients is desirable. On the other hand, SHIVE (**44**) has suggested that symptoms resulting from a limited supply of one element may be caused by toxic effects of another. The validity of this interpretation can be tested only by investigations which consider toxicity effects. If the viewpoint is accepted that the "balance" of micro-nutrient concentrations is the controlling or causal factor, then investigations relative to the effects of varied proportions of micro-nutrients are indispensable. In any event it is desirable to measure quantities of micro-nutrients within the plant as well as in the substrate, to clarify the "lack," "toxicity," "balance," or other relationship.

Summary

1. An experiment was designed to test the effects of manganese, copper, zinc, molybdenum, and iron deficiencies on the ascorbic-acid, riboflavin, and provitamin-A content of tomato fruits. The criteria of a micro-nutrient deficiency included measurements of growth and fruitfulness, development of deficiency symptoms, and chemical analyses of both leaflets and fruits. All data were reduced and analyzed by statistical methods.

2. An apparatus made from Pyrex glass is described for use in micro-nutri-

ent studies in the greenhouse when it is desired to grow plants to maturity and to test the effects of a number of treatments in replicated cultures.

3. External deficiency symptoms of vegetative parts are described for plants deficient in manganese, copper, zinc, and iron. A characteristic manganese-deficiency symptom which occurs in tomato fruits is described. A possible deficiency symptom shown by fruits with respect to zinc is also described.

4. Deficiencies of manganese, copper, zinc, and iron resulted in considerably less growth and fruitfulness than was measurable in control plants. The growth and fruitfulness of plants grown with a limited supply of molybdenum are comparable with those of control plants.

5. In general, chemical analyses of leaflets and fruits from plants grown with limited supplies of manganese, iron, zinc, and molybdenum showed significantly smaller quantities of the respective element than did the control plants. Development of the symptoms associated with a particular deficiency with respect to their position on the plant was closely correlated with the distribution of the element in the plant. The latter was determined by chemical analyses of leaflets from the top, middle, and lower thirds of the plant.

6. The ascorbic-acid content of fruits was not affected by limiting the supply of manganese, zinc, copper, and molybdenum, although growth and fruitfulness were seriously affected by a lack of manganese, zinc, and copper. Fruits from iron-deficient cultures contained 30 per cent more ascorbic acid than did control fruits on a fresh-weight basis. The quantity of fruit produced by iron-deficient plants, however, was significantly less than that of control plants.

7. Slight variations in riboflavin content of fruits could be correlated with the effect of micro-nutrient deficiencies. The magnitude of these variations was small and of no practical significance. The data show a pronounced effect on riboflavin content of variations in climatic environmental conditions such as occur with different positions in the greenhouse and with a different date of fruit maturation.

8. Provitamin-A content of the fruits was not significantly affected by a limited supply of the micro-nutrients. No significant effect of environmental factors was obtained with respect to this vitamin.

9. A discussion of the possible interpretation of these results is included.

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EFFECTS OF SOLVENT UPON ABSORPTION SPECTRA OF CHLOROPHYLLS *A* AND *B;* THEIR ULTRAVIOLET ABSORPTION SPECTRA IN ETHER SOLUTION[1]

D. G. HARRIS AND F. P. ZSCHEILE[2]

Introduction

Spectroscopic observations on preparations of chlorophyll components have been employed most often as a test for purity. In the case of extracts from natural sources, the spectra of the components are often used as analytical standards. In both cases the choice of solvent is usually made because of desirable physical or chemical properties or because of adaptability of the solvent to extraction of the particular plant material studied.

It appears desirable at this time, when more accurate applications of spectroscopic methods are being made to the analysis of plant material for chlorophyll (15, 5, 4), to have more complete knowledge of the spectra of the chlorophyll components in different solvents. Such information will facilitate a choice of solvents that is advantageous from the spectroscopic viewpoint.

The physical condition of chlorophyll in the living green leaf has been studied from numerous points of view (10, 16, 20). Many theories make use of the positions of the absorption maxima in the spectrum of the leaf in comparison with those for known preparations or solutions. The work outlined in this paper should be of some usefulness to those who would interpret the relation of spectrum to physical condition within the plant.

Experimentation

Purification of pigments

The method employed for the isolation of chlorophyll components *a* and *b* was essentially that described by Zscheile and Comar (29), with a few slight modifications. Magnesium carbonate was employed instead of calcium carbonate to neutralize plant acids in the extraction mixture. This minimizes the possibility of substitution of calcium for magnesium in the chlorophyll molecule (19).

For the series of preparations made during this study, the sources of chlorophyll were market spinach purchased at various seasons throughout the year and young corn seedlings grown in the greenhouse. It was found that the relative concentrations of carotenes to carotenols[3] varied among the individual lots of spinach, and the procedure for removing them from chlorophyll was varied accordingly. When the precipitated chlorophyll (29) is shown by the phase test to contain carotenoids, the following is added to the earlier procedure: The crude precipitated chlorophyll, after removal of the carotenes by washing with petroleum ether, is added (with accompanying sucrose) to a mixture of 25 ml. of petroleum ether and 15–25 ml. of ethyl ether. After complete solution of the chlorophyll, the volume is made to 1 liter with petroleum ether. Three washings with distilled water remove the sucrose. The carotenols still present are completely removed by washing twice with 200 ml. of 80 per cent, thrice with 85 per cent, and twice with 90 per cent aqueous methanol. The chlorophylls are then precipitated a second time and washed with petroleum ether. No carotenoids should now be

[1] Journal Paper no. 82, Purdue University Agricultural Experiment Station.

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[3] The use of this nomenclature was explained recently (27).

present in the crude chlorophyll, and the chromatographic separation of components *a* from *b* follows the earlier method (29).

Preparations of the components by use of these modifications have yielded ether solutions with spectroscopic properties essentially identical with those determined by ZSCHEILE and COMAR.

PREPARATION OF SOLUTIONS AND SPECTROSCOPIC STUDY

After the mechanical removal of the portion of the sucrose adsorption column containing the chlorophyll component to be studied, the pigment was eluted by various solvents. Spectroscopic observations were made as soon as possible. The chlorophyll was never dried in this procedure (29); the method of elution from the column thus resembles that employed by EGLE (6) for a study of Kundt's rule. The traces of ether and petroleum ether remaining on the column, which was almost dry, are regarded as insufficient to affect the results. In the case of chlorophyll *b* in cyclohexane, the addition of a small amount of ethyl ether was necessary for elution, after which the solution was washed with water.

Acetone and methanol were purified as previously described (25). Ethyl ether was distilled after standing over solid sodium hydroxide and was free of peroxide by the vanadic-acid test of JORISSEN (2). Methyl oleate was prepared from oleic acid and methanol according to the method of ARMSTRONG, ALLEN, and MOORE (1). The yellow color was removed from this ester by vacuum distillation. Commercial olive oil was filtered through a column of sodium aluminum silicate adsorbent (11) to remove colored impurities. The remaining solvents were purified according to methods described by WEISSBERGER and PROSKAUER (23).

The solvents were divided into two groups so that the data for six solvents could be obtained within 30 hours after the preparation had been completed. The solutions in which more rapid decomposition was expected were studied first. For the first group of solvents the order was methanol, acetone, benzene, cyclohexane, carbon tetrachloride, and isopropyl ether; for the second group it was methyl oleate, olive oil, butanol-1, 2-methyl propanol-1, 2-ethyl hexanol-1, and 1, 4-dioxane. These solvents were chosen for various reasons. Since many disagreements exist in the literature on the positions of absorption bands, certain solvents were chosen to provide further tests of Kundt's rule. Other factors, such as viscosity, dipole moment, dielectric constant, and boiling point, were considered. Some solvents were used because of analytical importance; certain others because of their possible similarity to certain lipoid materials of the plastid.

Results in the visible region were obtained by the same spectroscopic instrument and methods previously described (29). The temperature of the solution was not controlled but occasional measurements during the progress of the work indicated a narrow range of 24°–26° C. Since 1-cm. absorption cells were used throughout, the concentrations, though not known accurately, were of comparable magnitude. Thus, results should be comparable with those obtained by EGLE (6) so far as uniformity of concentration is concerned.

For the ultraviolet measurements below 3260 A, a quartz hydrogen arc of the MUNCH type (17) was employed as a source of ultraviolet radiation. An incandescent source was used above 3260

A. Values were plotted at intervals of 20 A. Spectral regions isolated (9) and slit widths were as follows:

Wavelength	Slit width	Spectral region isolated
2660–3260 A	0.17 mm.	6–11 A
3260–3360	0.20	13–15
3360–3500	0.10	7–8
3500–4100	0.06	5–9

Results

Absorption spectra in different solvents

Many difficulties would have been encountered had an attempt been made to present the data as specific absorption coefficients. To determine the numerical values of such coefficients, the concentration of the solution must be known. The weight of the dissolved chlorophyll would have been difficult to obtain in many cases, owing to the difficulty of quantitative evaporation of the solvent. With some solvents, quantitative transfer to ether and subsequent spectroscopic analysis (5) would be possible, but since the main objective of this study was to determine the positions and relative heights of the absorption bands in relation to various properties of the solvents, the values of relative absorption were plotted against wavelengths. Absorption = log I_0/I, as defined by Hogness and Potter (8), where I_0 and I are the intensities of radiation transmitted by the solvent-filled and solution-filled cells, respectively.

The absorption curves for chlorophyll *a* are presented in figures 1 and 3. All the observed absorption values were multiplied by appropriate factors, chosen so that the curves would all pass through one point on the graph. The wavelength of this point, 5050 A, with relative absorption value of 0.165 (absorption value chosen arbitrarily for convenience only), was chosen because it has been shown that this region of the curve is very sensitive to degradation products in the case of ethyl ether solution and because the curves obtained by Zscheile and Comar (29) for ethyl ether solutions of the chlorophyll components were plotted on the same basis for comparison. The major red maxima are plotted slightly higher than those previously published because of the slightly higher ratio values obtained on these recent preparations. This makes the curves more representative of the latest preparations. Whether the cause of this difference lies in the red or in the green, or in both, is not known. The values on the chlorophyll *a* curve for ether (29) were multiplied by a factor such that the same absorption value would result at 5050 A. The absorption was quite low at this wavelength for all the solvents studied.

Owing to the smaller quantities of component *b* available for study, only five solvents were compared. Curves presented in figure 4 were obtained by multiplying by suitable factors to provide a common point with the ether curve at 5200 A. For comparison, the ether curve (29) has been plotted also. To maintain the same relative heights of the curves for chlorophylls *a* and *b*, the chlorophyll *b* curve was also multiplied by the same factor as used for component *a* to give an absorption value of 0.263 at 5200 A, where degradation products of chlorophyll *b* are easily detected in ether solutions.

The positions of the two principal maxima in the red and blue regions of the spectrum of chlorophyll *a* in each of the thirteen solvents are presented in table 1, with the solvents arranged according to index of refraction. The R_a value has been useful to indicate decomposition in ether solutions (29). A corresponding value for each solvent has been calculated.

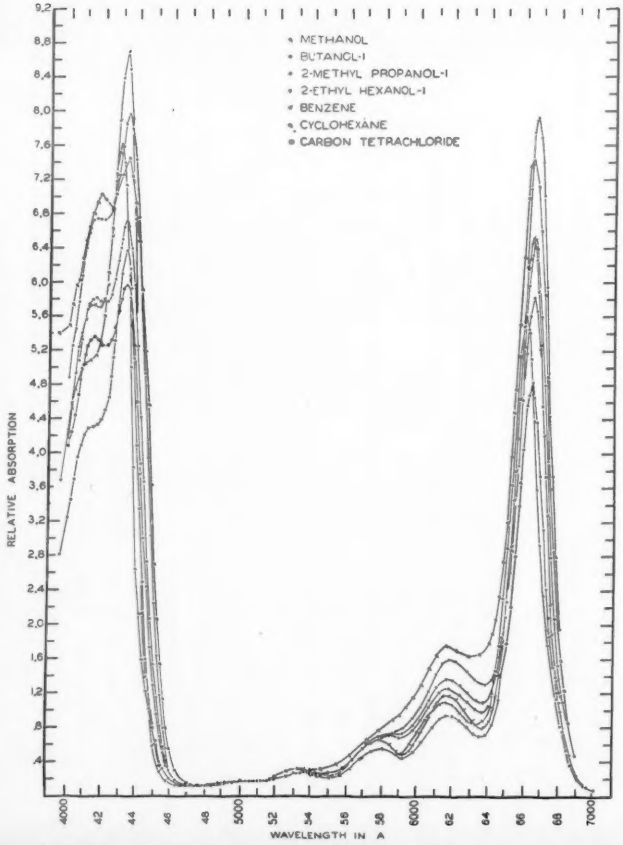

FIG. 1.—Absorption spectra of chlorophyll *a* solutions

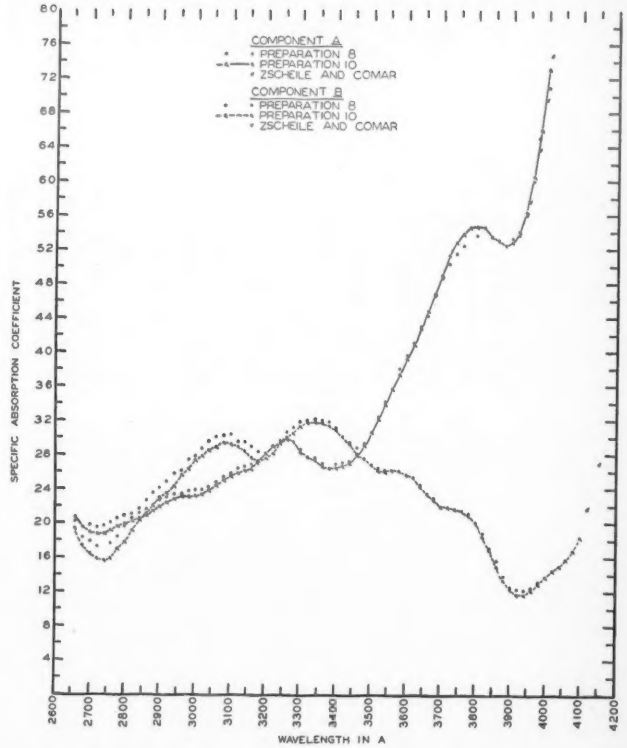

FIG. 2.—Ultraviolet absorption spectra of chlorophylls *a* and *b* in ethyl ether solution.

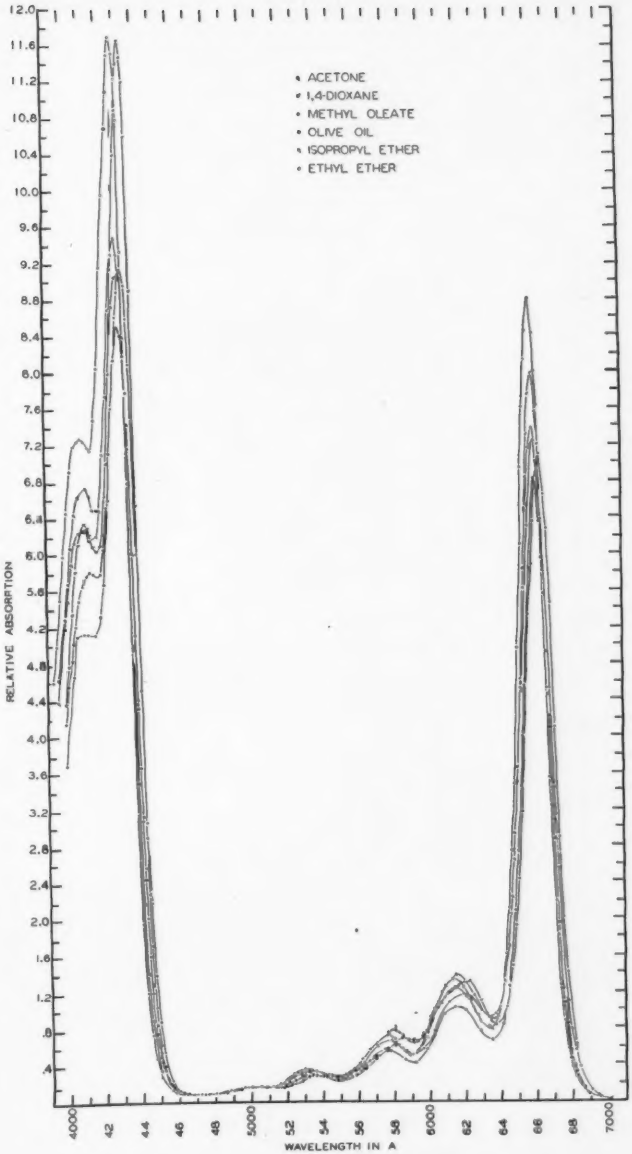

FIG. 3.—Absorption spectra of chlorophyll *a* solutions

FIG. 1.—Absorption spectra of chlorophyll *a* solutions

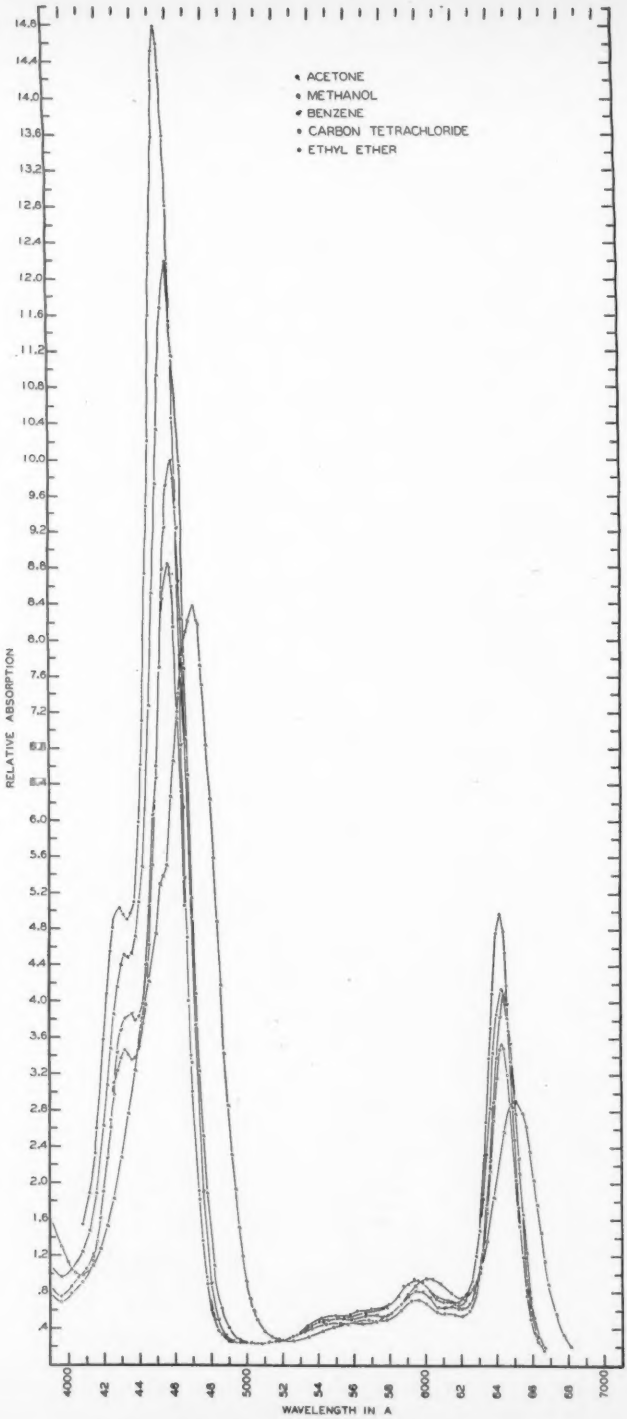

FIG. 4.—Absorption spectra of chlorophyll *b* solutions

In this comparison of the spectra in different solvents, no use is made of specific absorption coefficients, designated in other work from this laboratory by "$\propto$." However, the R values of tables 1 and 2 calculated from the relative absorption (29). A similar value, R'_a, for the blue maximum permits a useful comparison. The ratio of R'_a to R_a expresses the ratio of absorption intensity at the blue maximum to that at the red maximum. These calculations are presented in table 1.

TABLE 1

ABSORPTION MAXIMA OF CHLOROPHYLL *a* IN VARIOUS SOLVENTS

Solvent	Index of refraction (20° C.)	Major red maximum (A)	Minor red maximum (A)	Major blue maximum (A)	Minor blue maximum (A)	R_a*	R'_a†	$\frac{R'_a}{R_a}$
Methanol	1.33	6640	6165	4340	4180	45.0	45.1	1.00
Ethyl ether	1.35	6600	6140	4290	4100	52.7	70.2	1.33
Acetone	1.36	6615	6155	4315	4120	43.9	55.2	1.26
Isopropyl ether	1.37	6610	6145	4300	4115	44.6	57.5	1.29
2-methyl propanol-1	1.39	6645	6175	4330	4150	35.2	36.1	1.02
Butanol-1	1.40	6650	6175	4325	4155	39.4	40.7	1.03
1, 4-dioxane	1.42	6610	6215	4330	4110	48.3	70.7	1.46
Cyclohexane	1.43	6600	6160	4295	4100	33.9	46.1	1.36
2-ethyl hexanol-1	1.43	6665	6190	4340	4160	48.0	48.2	1.00
Methyl oleate	1.46	6620	6160	4315	4115	41.3	51.5	1.25
Carbon tetrachloride	1.46	6635	6180	4325	4135	29.3	38.6	1.32
Olive oil	1.48	6645	6200	4330	4140	43.0	55.5	1.29
Benzene	1.50	6640	6175	4325	4135	39.5	52.7	1.33

*$R_a = \frac{\propto \text{ red maximum}}{\propto 5050}$. †$R'_a = \frac{\propto \text{ blue maximum}}{\propto 5050}$.

TABLE 2

ABSORPTION MAXIMA OF CHLOROPHYLL *b* IN VARIOUS SOLVENTS

Solvent	Index of refraction (20° C.)	Major red maximum (A)	Minor red maximum (A)	Major blue maximum (A)	Minor blue maximum (A)	R_b*	R'_b†	$\frac{R'_b}{R_b}$
Methanol	1.33	6510	6025	4710	4540	11.3	32.2	2.85
Ethyl ether	1.35	6425	5940	4530	4285‡	18.9	56.2	2.98
Acetone	1.36	6435	5950	4560	4315	15.9	46.9	2.95
Carbon tetrachloride	1.46	6440	5950	4570	4320	13.4	34.0	2.54
Benzene	1.50	6450	5965	4590	4350	15.7	38.4	2.45

*$R_b = \frac{\propto \text{ red maximum}}{\propto 5200}$. †$R'_b = \frac{\propto \text{ blue maximum}}{\propto 5200}$.

‡Attention is called to a typographical error of 15 A in the position of this maximum as reported earlier (29).

will have the same numerical values as though they had been derived from specific absorption coefficients, since the validity of Beer's law is the basis of all these comparisons. For brevity, no distinction is made between the R values calculated here and those defined earlier Similar data for component *b* in five solvents are presented in table 2.

CRITERIA OF PURITY

The spectroscopic criteria of purity presented by ZSCHEILE and COMAR for ether solutions (29) were used in this

study. A small portion of each preparation was eluted from the column with ethyl ether and tested to establish the purity of the chlorophyll preparation as a whole. For chlorophyll *a* (experiment 7), R_a was 53.8 in ether. This preparation was made from corn leaves and was studied in methanol, acetone, isopropyl ether, cyclohexane, carbon tetrachloride, and benzene solutions. Component *a* (experiment 2), from spinach leaves, was studied in 1, 4-dioxane, 2-methyl propanol-1, butanol-1, 2-ethyl hexanol-1, methyl oleate, and olive oil. Its R_a value was 46.8 in ether. This is not as good a preparation as the other, but time did not permit a repetition on a better preparation. Therefore significance should not be attached to very small spectroscopic differences between curves for solutions of these two preparations of component *a* in correspondingly different solvents because of the difference in purity.

For preparation 8, used in the ultraviolet study, R_a was 52.6 and R_b was 18.7 in ether, which is in good agreement with preparations studied previously (**29**). In the case of preparation 10, higher values were obtained. These ratio values were measured by two methods: (1) with the customary 1-cm. cell and appropriate dilutions of 51- and 11-fold for *a* and *b* respectively; and (2) with the same concentration throughout but using a cell length of 1 cm. for the wavelengths 6600 and 6425 A and 12 cm. for wavelengths 5050 and 5200 A. The solutions employed are thus quite dilute. The log values ranged from 0.81 to 0.18 in the case of *a* and from 0.30 to 0.52 for *b*, the latter being optimum and the former only slightly outside the working range customarily used in this laboratory (**29**). The R_a values obtained by methods (1) and (2) were 53.4 and 53.0, respectively, and the corresponding R_b values were 19.2 and 19.5. These R_a values agree well with the highest value of 53.3, reported earlier (**29**) from a corn leaf sample. These R_b values are *ca.* 5 per cent higher than the average of three earlier values (**29**). ZSCHEILE, COMAR, and MACKINNEY (**30**) recently reported absorption values obtained on a dried sample of chlorophyll *b* prepared by MACKINNEY, from which R_b values of 18.0–18.2 may be calculated. These two preparations were made from spinach leaves. All absorption spectra of figure 4 are of chlorophyll *b* (experiment 8). In calculating the concentrations from absorption values at the red maxima, specific absorption coefficients of 103.0 (6600 A) and 58.0 (6425 A) for *a* and *b*, respectively, were used for preparation 10 because of the higher ratio values, instead of corresponding average values of 102.1 and 56.8 (**29**) as used for preparation 8. The difference between the R values found for preparation 10 and earlier values was thus arbitrarily divided between a possible increase in the red and a decrease in the green regions, since a definite decision on the exact cause of the increased R values was not possible.

DRIED CHLOROPHYLL

An effort was made to dry chlorophyll *a* very rapidly in the absence of a glass surface by evaporation of a small stream of an ether solution (R_a = 51.1) injected into an evacuated glass chamber through a capillary tube. Some solution struck the sides of the vessel and therefore some evaporation occurred from the glass surface, which previous work indicated should be minimized (**29**). An R_a value of 42.9 was observed immediately after drying in this manner.

During the early part of a certain preparation of chlorophyll *a* from corn seedlings, an unusual tendency was noted

for the chlorophyll to precipitate.[4] Precipitation started after the first washing of the petroleum ether solution with 80 per cent aqueous methanol and was almost complete after the third washing. After filtration and chromatography on sucrose, the phase test on the chlorophyll of the lower portion of the column indicated the presence of carotenoids. After solution in an ether-petroleum ether mixture, it was washed repeatedly with 80 per cent methanol and finally with 90 per cent methanol. Following two washings with water, the chlorophyll *a* precipitated in leaflike flakes of unusually large size. A portion of the material was removed without drying, dissolved in ether, and tested spectroscopically. The R_a value was 53.0. After drying under reduced pressure for 1 hour, an R_a value of 55.7 was observed; after further drying under high vacuum, the R_a value had decreased to 53.9, indicating a small increase rather than a decrease on drying. This was unusual in our experience, since many previous efforts to dry chlorophyll with retention of a high R_a value had failed (**29**). The ratio of the maximum at 6600 A to that at 4100 A was identical with the average reported (**29**) on undried samples. The cause for the increase of R_a on drying this sample is not known. This sample (experiment 6) was small in amount and permitted only limited study of its properties during cold storage. After 9 weeks, the R_a value of the dried portion had decreased to 48.9, whereas a portion kept at the same temperature in ether solution still had a very high R_a value, 54.0.

It is thus possible to dry chlorophyll *a* with retention of the spectroscopic properties it had before drying (at least so far as indicated by the R_a value). The optimum conditions have not been clarified, however, and rigid spectroscopic control should always be relied upon as a criterion of purity. In our experience chlorophyll is most stable when kept in ether solution at a low temperature. Values of $\frac{R'_a}{R_a}$ for ether solutions of a dried sample prepared by MACKINNEY and studied in this laboratory (**30**) approach very closely the value reported here.

The addition of a small amount of hydrogen peroxide to an ether solution of chlorophyll *a* (R_a = 51.1) reduced the R_a value to 46.0 after 2 hours. The ratio of the absorption at 4290 to 6600 A checked average values reported earlier (**29**). The absorption at 6600 A was unchanged but that at 5050 A was increased 11.7 per cent, causing the observed decrease in R_a. This change was not due simply to the effect of the acidic condition, since absorption at 6600 A would then have decreased.

ULTRAVIOLET SPECTRA

The ultraviolet absorption data are plotted in figure 2, as the specific absorption coefficient (**29**) versus wavelength. The concentrations were determined spectroscopically at the red maxima on solutions eluted directly from the adsorption columns. Maxima or shelves in the curves were found at the following wavelengths: 2820, 2960, 3120, 3260, and 3800 for chlorophyll *a;* 2900, 3060, 3250, 3340, 3570, and 3750 for chlorophyll *b*. Minima have wavelengths as follows: 2730, 3380, and 3880 A for component *a;* 2740, 3180, and 3930 A for component *b*.

Discussion

The group of solvents in which chlorophyll *a* was studied includes three ethers, four alcohols, one ester, a mixture of

[4] A similar tendency for early precipitation was observed previously by C. L. COMAR in the case of a preparation from spinach made in this laboratory.

esters, aromatic solvents (both saturated and unsaturated), a ketone, and one with a very symmetrical molecule, carbon tetrachloride. These solvents cover a considerable range of polarity (as indicated by the dielectric constants) and refractive index, as well as other physical properties such as boiling point, specific gravity, and internal pressure. Significant correlations with spectroscopic properties could be found only with the first two of these physical properties, discussed in the following paragraphs.

KUNDT'S RULE

Kundt's rule, that the absorption band of a solute dissolved in a colorless solvent of high refractive index is shifted toward longer wavelengths compared with the corresponding absorption band of the same solute in another colorless solvent of lower refractive index, has often been studied in relation to the red maximum of chlorophyll (**3, 6, 10, 12, 22**). Certain controversial and historical aspects of this question have been discussed by MACKINNEY (**12, 13**).

CHLOROPHYLL *a*.—From table 1 the following conclusions may be drawn, considering all the bands listed:

1. When all solvents are considered together, no correlation exists between position of maxima and refractive index of the solvent.
2. Among the four alcohols studied, the red maxima follow Kundt's rule; the blue maxima do not.
3. Among the ethers, differences are small but not contradictory to Kundt's rule, within the limits of error.
4. The rule is followed between ethyl ether and acetone, but if isopropyl ether is included in the comparison the rule is not obeyed.
5. Among the two hydrocarbons, cyclohexane and benzene, all four maxima follow the rule.
6. Among the ester solvents, methyl oleate and olive oil, all four maxima follow the rule.

It may be noted that when two solvents with the same refractive indices are compared, as cyclohexane with 2-ethyl-hexanol-1, and methyl oleate with carbon tetrachloride, great differences occur between the wavelengths of corresponding maxima. In some cases this difference is equal to the entire range found for the thirteen solvents studied. With such pairs of solvents, great differences exist in their chemical nature. With such a pair as 2-methyl propanol-1 and butanol-1, with nearly the same refractive index but similar chemical properties, wavelengths of maxima are essentially identical.

CHLOROPHYLL *b*.—The more limited number of solvents in which component *b* was studied prevents comparisons as extensive as those made for component *a*. With the exception of methanol, Kundt's rule is obeyed fairly well, although differences between acetone and carbon tetrachloride are very slight and not so great as was the case for component *a*. Comparisons between ethyl ether and acetone are similar for both components. Differences between carbon tetrachloride and benzene are greater than was the case for chlorophyll *a*. These two solvents have about equal dielectric constants. No better correlations exist between dielectric constant and wavelengths of maxima than was the case for component *a*.

Discrepancies in wavelengths prevent a good comparison with BIERMACHER'S work (**3**) on chlorophyll *a*, but in the case of the *b* component his data agree with those presented here so far as relative positions of maxima are concerned.

The conclusion of HUBERT (**10**) that the band shifts to shorter wavelengths in polar media is not substantiated for the case of methanol in comparison with ether, acetone, and benzene. In general the data for the four alcohols agree with the observations of EGLE (**6**), that in comparison with other solvents studied

the bands occur at longer wavelengths than would be expected from Kundt's rule. The relationship between ethyl ether and benzene solutions also agrees with his report. Certain causes for disagreement among various workers on this subject have been discussed previously (27).

Thus, the statement of WAKKIE (22) that Kundt's rule is obeyed when restricted to substances which have certain hydrophilic groups in common is upheld to a limited extent; it is also evident that Kundt's rule cannot be applied to chlorophyll solutions in general. The limited data presented here are favorable to Kundt's rule only when applied to solvents of similar chemical nature.

ANALYTICAL APPLICATIONS

The ratio values of tables 1 and 2 and the absorption curves of figures 1, 3, 4 permit numerous comparisons having analytical applications. In comparing the ratios of the absorption values at the blue maxima with those at the red maxima, a clear distinction exists for chlorophyll *a* between the four alcohols as a group and the remaining solvents. In the alcoholic solutions the blue and red maxima are of equal heights, whereas in all the other solvents the blue maximum is considerably higher than the red. The data for methanol were obtained on a preparation different from that used for the other three alcohols. This similarity between alcoholic solutions would thus appear not to be a property peculiar to a single preparation.

For methanol solutions MACKINNEY (15) and STRAIN and MANNING (21) reported a slightly higher (*ca.* 4 per cent) absorption at the red maximum of component *a* than at the blue maximum. The relative height of the lowest minimum for methanol agrees better with STRAIN and MANNING'S (21) curve for component *a* but better with MACKINNEY'S data (15) for component *b*. There are great discrepancies between results reported here and those of RABINOWITCH and WEISS (18), not only in relative heights of bands but also in wavelengths of maxima and presence or absence of bands. The $\frac{R'_a}{R_a}$ and $\frac{R'_b}{R_b}$ values calculated from MACKINNEY'S data (15) are 0.96 and 2.82, respectively, compared with corresponding values of 1.00 and 2.85 of tables 1 and 2. These data indicate good agreement, but differences in wavelength in the blue region throw some doubt on the validity of this comparison. Uncertainty arises because MACKINNEY has reported (15) wavelengths as "near maximum."

Corresponding values for anhydrous ether solutions calculated from previous data (29) are 10 per cent higher than those from MACKINNEY'S values (15). Here again, wavelengths may not be directly comparable for the blue region, although agreement is excellent in the red.

For acetone solutions the values of $\frac{R'_a}{R_a}$ and $\frac{R'_b}{R_b}$ agree well with those calculated from MACKINNEY'S data (14). Values from his data vary with source as follows: $\frac{R'_a}{R_a}$ from 1.22 to 1.30, average 1.26; $\frac{R'_b}{R_b}$ from 2.61 to 2.98, average 2.75. Corresponding values reported here are 1.26 and 2.95, respectively.

The curves for benzene solutions do not agree with those determined by HAUSSER (7) and WINTERSTEIN and STEIN (24) when the relative heights of maxima are considered. HAUSSER'S curve for component *a* has an extra band near 5500 A. The ratios of blue to red maxima reported here are considerably lower than those of WINTERSTEIN and STEIN (24) and closer to those calculated from data

of MACKINNEY (14). The wavelengths of maxima are in good agreement with those reported by MACKINNEY (14).

In the case of component *b*, the solutions with solvents containing oxygen have higher values of $\frac{R'_b}{R_b}$ than those of the other two solvents, which is not true with the values of $\frac{R'_a}{R_a}$. The blue region is of importance to characterize the components but has less analytical importance than the red region because of the interference of carotenoids in most practical applications.

In spectrophotometric analysis it is generally desirable to choose a solvent in which the substance has the most sharp, intense, and well-defined absorption bands, although other considerations are also necessary for practical reasons. Sharp intense bands increase the sensitivity of analysis and facilitate more accurate tests for identification and purity of samples. Ethyl ether has been used in analytical applications (5, 4), and it is clear that the R_a and R_b values for this solvent are higher than for other solvents examined, although several others have values of comparable magnitude. The R'_a and R'_b values are also very high. Corresponding values of R_a and R'_a for 1, 4-dioxane are nearly the same.

The curve for component *a* in 1, 4-dioxane, although having very sharp major maxima in the blue and red regions, does not resolve the band near 5850 A clearly. The band at 4110 A is most clearly resolved in this solvent. The spectra of acetone solutions do not have as sharp bands as those of ethyl ether solutions, although acetone is not an unfavorable solvent from this viewpoint. Aqueous acetone has been used as an analytical solvent (15). Among the alcohols studied, 2-ethyl hexanol-1 has the highest values of R_a and R'_a. In general, spectra of the alcoholic solutions do not have the minor bands well resolved. Spectra of methanolic solutions of both components are particularly deficient in structural detail.

ULTRAVIOLET SPECTRA

The ultraviolet region was not studied as intensively as the visible region because its analytical importance is minor, effects of contaminants are greater, and it has little application to photosynthesis compared with the visible region. The general characteristics of the ultraviolet curves agree well in wavelength with those found earlier for chlorophylls *a* (26, 28) and *b* (26). The ultraviolet spectra determined by HAUSSER (7) and by WINTERSTEIN and STEIN (24) for benzene solutions are lacking in structural detail but have somewhat the same general shape as the curves reported here for ether solutions.

Summary

1. Solutions of chlorophyll *a* were prepared in thirteen solvents and of chlorophyll *b* in five solvents by direct elution from sucrose adsorption columns.

2. Absorption spectra for each solvent are reported as measured spectrophotoelectrically in the visible region.

3. Spectra for the ultraviolet region to 2650 A were determined for solutions in ethyl ether.

4. A preparation of dried chlorophyll *a* was made which retained excellent spectroscopic properties.

5. The application of Kundt's rule to chlorophyll solutions is discussed, as well as certain relations of spectroscopic properties and solvents to analytical applications.

It is a pleasure to acknowledge the aid of C. L. COMAR in the first preparation employed in this work, that of ARTHUR M. BRUNSON and RALPH LINCOLN in supplying the corn seedlings as a source of part of the chlorophyll, and the assistance of L. MCALISTER in many of the calculations.

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CYTO-GENETIC CONSEQUENCES OF X-RAY TREATMENT OF POLLEN IN PETUNIA

CHARLES M. RICK

Introduction

Artificial stimulation of the haploid egg is attracting ever-increasing interest. The usefulness, both economic and theoretical, of a dependable method for producing haploid organisms, has been pointed out by EAST (6), BLAKESLEE (2), and others. The possibilities of attaining this goal by the use of chemicals are already being explored (23).

Some investigators have succeeded in raising the frequency of haploids by various techniques. Certain of these methods have also yielded maternal diploids which are suspected of having originated by doubling of the chromosome number in a haploid egg. In this way pure lines would be produced with greater ease than by way of haploids. No attempt is made here to review all the pertinent literature, since IVANOV (10) has adequately surveyed the subject up to 1938. Polyembryony has been the source of a great number of plant haploids (24), but their production by this technique is naturally limited to those species in which there is a fair frequency of multiple seedlings, including haploids. Interspecific hybridization also accounts for many haploids. JØRGENSEN (12) reported the high yield of 20 per cent haploids in thirty-five plants produced by the cross *Solanum nigrum* ($2n = 72$) × *S. luteum* ($2n = 48$). KIHARA (14) found 37.5 per cent haploids in *Triticum monococcum* from flowers emasculated 9 days before the application of normal pollen, but this figure was based on a population of only eight plants.

The use of X-rayed pollen was found by KATAYAMA (13) to yield 17.6 per cent haploids (in a total of ninety-one plants) in a line of *T. monococcum* which without treatment produced only 0.48 per cent haploids. In the varieties *humilis* and *texana* of *Nicotiana rustica*, IVANOV (10) secured four haploids and seven maternal diploids by means of X-rayed pollen. The failure of X-rayed pollen to stimulate the production of haploids in *Datura* is reported by BLAKESLEE (2). The results of similar treatment in the present experiment were negative. Certain aspects of this study of the cytologically aberrant plants resulting from the X-ray treatment have already been published in abstract form (20).

STEBBINS (21) has pointed out the frequent misuse of the term parthenogenesis in this type of experiment. In the functional sense parthenogenesis implies natural development of the egg without external stimulation. It is more accurate to designate as haploid, or reduced pseudogamy, cases where development of the haploid egg is a response to the activity of pollen which is incapable of fertilization. It is possible, as already mentioned, that some external stimulus might cause the doubling of the egg chromosomes and the subsequent development of a diploid embryo. In this paper the term haploid-diploid pseudogamy is arbitrarily chosen to indicate this phenomenon.

Investigation

HAPLOID PSEUDOGAMY

Anthers of *Petunia* were collected immediately before anthesis and stored in gelatine capsules. The source of X-rays was a Wappler clinical unit operated at 200 kv. and 10 ma. Filters of $\frac{1}{4}$ mm. copper and $\frac{1}{4}$ mm. aluminum were employed. All anthers were irradiated in capsules at a distance of 10 cm. from the

tungsten anticathode, at which distance the intensity of irradiation was approximately 800 r per minute. By varying the lengths of exposure, doses from 10,000 to 100,000 r were achieved. Shortly after this treatment, the pollen was applied to stigmas of previously emasculated flowers. Simultaneously some stigmas were dusted with untreated pollen and others were left unpollinated. Approximately forty flowers were pollinated at each dose; a total of about 700 pollinations was made. Seeds from all of these treatments were harvested, and, starting with lots from pollen which had received 100,000 r and proceeding toward less severe treatments, seeds were sown and plants grown to maturity for further study. All sowings were made on nutrient agar under aseptic conditions in order to carry through the tender seedling stage as many as possible of the weaker, more aberrant types.

If by means of this treatment the haploid egg cell were to be stimulated to develop pseudogamously, it would give rise to a haploid plant. Accordingly all plants produced by pollen surviving the highest doses were examined cytologically. This search for haploids was accomplished by examination of metaphase chromosome figures in root tips, which of course would also reveal any gross chromosomal aberrations. The root tips were fixed, imbedded in paraffin, sectioned, and stained with iodine-crystal violet in the usual fashion. RANDOLPH'S method (18) of mounting root tips was found excellent for this purpose.

Prior to germination, no satisfactory criterion of the most effective X-ray dose could be found. Even pollen which had received 100,000 r stimulated considerable development of the ovary and production of some seeds, which, although inviable, were very difficult to distinguish from viable seeds. Furthermore, there was great fluctuation in the number of seeds produced and in the size of the mature fruit.

As noted in table 1, pollen could effect fertilization after doses as high as 50,000 r. There can be little doubt that treated pollen functioned here, since one of the plants produced showed a typical X-ray-induced chromosomal aberration. Although the results were too diverse to permit the establishment of a relation between dose and germination, it is possible to estimate the half-kill doses. Germination was reduced to half the level of untreated material at about 16,000 r. The ratio of the numbers of plants produced per flowers pollinated was reduced to half at approximately 9,000 r. The highest frequency of cytologically aberrant plants, 18.5 per cent, was produced by pollen which had been exposed to 30,000 r.

No haploids were found in the sample of 378 plants whose root tips were examined. This sample included all plants from the 25,000 to 100,000 r lots, and some of the plants from lower doses, which survived until time of chromosome examination.

TABLE 1

SUMMARY OF X-RAY EFFECTS

Source of pollen	No. of flowers pollinated	Germination (%)	Mean no. plants per pollination	Diploid	Aberrant*	Aberrant (%)
(Not pollinated)	25	.....	.....	.....	.....	.....
P117 untreated	61	53.3	64	All	.....	.....
P117 10,000 r	30	40.2	28.7	32	1	3.1
P117 20,000 r	35	17.7	13.2	67	9	11.8
P117 25,000 r	36	14.1	6.4	121	15	11.6
P117 30,000 r	61	7.0	0.82	22	4	18.5
P117 40,000 r	66	8.7	0.5	13	1	7.1
P117 50,000 r	69	4.9	0.32	12	1	7.7
P117 60,000 r	31	2.9	0.1	2	.....	0.0
P117 75,000 r	32	2.4	0.1	12	.....	0.0
P117 100,000 r	29	0.0	0.0	.....	.....	.....

* Under aberrant are listed the following types: $2n = 13$, $13 + 1f$, $14 + f$, $13 + 2f$, $12 + 2f$.

The failure of haploid plants to appear in this sample might be attributed to failure of the treatment to induce haploid pseudogamy. No haploids in *Petunia* have been reported in the literature, in spite of the frequent use of this genus in cyto-genetic research. If the frequency of natural occurrence of haploids can be taken to mean anything in regard to their artificial production (and this is borne out in the case of *T. monococcum*), then it is not unusual that no haploids were obtained in this experiment. Haploid-diploid pseudogamy may have occurred, thereby throwing all potential haploids into the diploid group through doubling of the chromosome number, although further results—discussed later—shed considerable doubt on this hypothesis. The other possibilities—that haploids were produced but died in early seedling stages or that the sample was too small to reveal any induced haploidy—obviously cannot be ruled out.

HAPLOID-DIPLOID PSEUDOGAMY

Diploids arising by haploid-diploid pseudogamy would be completely homozygous, and their progeny (by selfing) could be readily distinguished from the more variable progeny of diploids of any other origin when the X-rayed pollen has been applied to stigmas of plants heterozygous for a considerable number of characters. To reveal this mode of origin, the following plan was adopted as shown on opposite page.

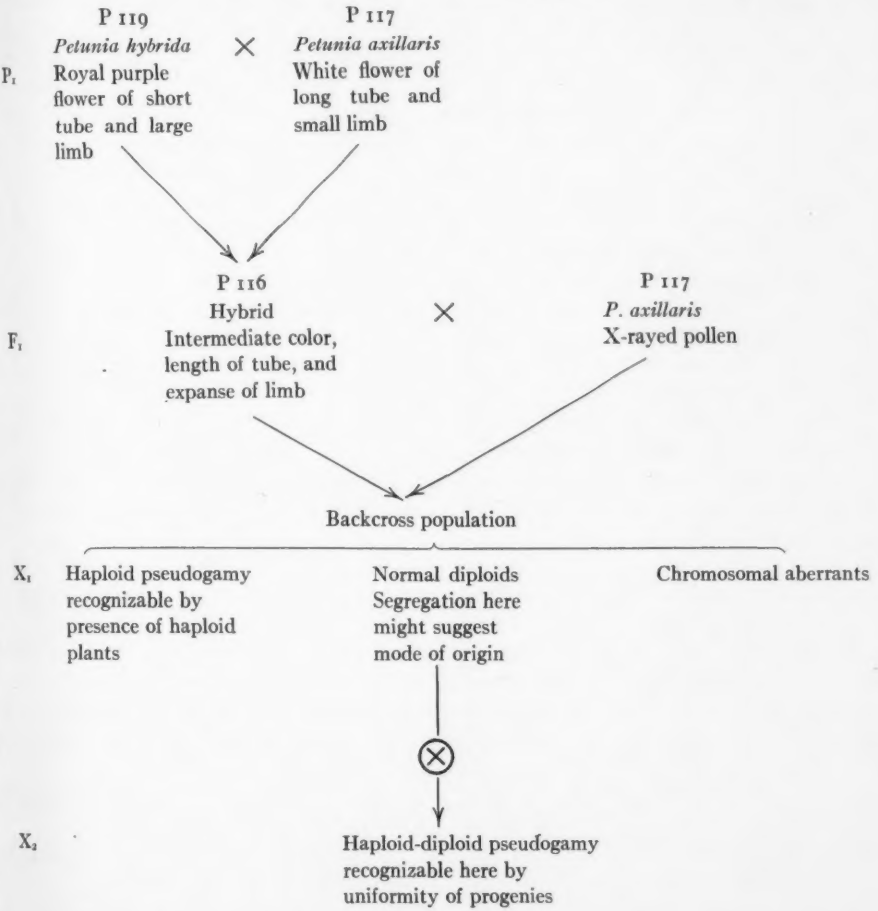
A somewhat similar plan was followed by EAST (7). Plants of *Fragaria vesca* heterozygous for two Mendelian factors were pollinated by *F. virginiana* and *F. chiloensis*. All plants obtained from this cross were typical *F. vesca*. Ratios obtained for the two factors were 19:5 and 16:12, suggesting that some individuals might have been produced by haploid-diploid pseudogamy. A more rigorous proof might have been obtained by selfing these plants and testing the next generation for uniformity, as proposed in the present experiment; but this EAST did not attempt owing to "certain technical difficulties."

In the experiments of EAST (7) and IVANOV (10), the pollen used to induce pseudogamy carried factors which would be manifest in the phenotypes of hybrid offspring. Thus it was possible to distinguish between true hybrids and maternal diploids. However, in neither case could a separation be made in the matroclinous group between those resulting from self-pollination and those resulting from apomixis—presumably haploid-diploid pseudogamy. Although the present experiment would have been improved by the use of X-rayed pollen carrying factors which would identify hybrid individuals in the offspring, an adequate pollen source of this type was not available.

Many genetic differences exist between P 119 and P 117, the parents of the hybrid used as receptor of the X-rayed pollen. The presence or absence of anthocyanin pigment in the limb of the corolla of these lines is determined by a single gene pair, according to results obtained in this study and to FERGUSON (8). In addition to this basic difference, an undetermined number of supplementary genes exist which modify the color variously in a range from pale lilac to deep royal purple. Variation was also noted in the coloration of the throat of the corolla. Male-sterility, a simple recessive character, also appeared in some of the F_2 populations. The differences in dimension of the flower corolla were inherited in the expected quantitative fashion. Of these dimensions, length of

tube and diameter of limb were most conveniently measured. Many other differences of a less tangible nature exist between P 119 and P 117.

All applications of the X-rayed pollen were made in the field, where every possible precaution against self-pollination and contamination was observed. The failure of the twenty-five unpollinated flowers to set any seed attests to some degree the conditions of the experiment. Yet, in view of the great number of pollinations attempted, occasional contamination could hardly be avoided. This was anticipated in the design of the experiment, which attempted to distinguish only completely homozygous individuals from those of hybrid origin, whether by X-rayed pollen or contamination. Nevertheless, the type of segregation obtained frequently indicated the type of hybrid origin. Contamination was encountered, and—especially when it occurred in the higher doses—it obscures the relation between X-ray dose and frequency of induced aberration.



For example, among the thirty-two ovaries developed after application of pollen treated with 75,000 r, none produced viable seed except one which gave rise to 14 plants—a fact which in itself suggests contamination. Of these fourteen, eleven survived to flowering; all were diploid; ten had colored flowers, one had white flowers. Obviously this could not represent a backcross family or a family arising out of haploid-diploid pseudogamy, for in either case a 1:1 ratio is expected. By noting segregations in other suspiciously large families, certain other diploids were dismissed from consideration as possible homozygotes.

A sample of forty-five diploids was selected for the tests of homozygosity. These included all diploids produced by pollen receiving doses of 40,000 to 75,000 r; also four families in the 25,000 and 30,000 r lots, each of which included some aberrant individuals and showed the expected 1:1 segregation for flower colors. Nineteen of the forty-five diploids were ruled out as contaminants because of aberrant segregation, as in the case of the 75,000 r lot already described.

The remaining twenty-six plants were self-pollinated and sample progenies grown for comparison with pure lines, etc., as described in the preceding section. Of these, twenty-two were found to segregate for some readily detectable qualitative character (for example, color of corolla or male-sterility). Dubious cases were reserved for flower measurement. Judging from the type of segregation (for example, those in which white offspring always appeared), the majority of the plants being tested arose from the intended backcross and few from contaminating pollen. The appearance of characters not present in either of the original parents could not be safely relied upon as a criterion of contamination, because some of these might be the expression of mutation induced by the X-radiation. The four remaining populations showed no such tangible variation. But the fact that each of these groups had only white corollas (that is, were homozygous recessive for the color-determining gene) would suggest that they were only the proportion of amphimictic families expected to be homozygous for the qualitative characters observed. This surmise was verified by measurements summarized in table 2.

These four families, together with five families of P 117, the pure line of *P. axillaris*, were grown under comparable conditions in the same field. It will be noted that the average standard deviation for the two dimensions of the lines being tested greatly exceeds that of the pure lines. Where in a few cases the standard deviation of one dimension in a test family does not significantly exceed the highest standard deviation for that character in the pure-line families, the standard deviation of the other dimension in that test family does so. Thus it must be concluded that no evidence of haploid-diploid pseudogamy was encountered in this experiment.

Again, as in the search for haploids, it must be admitted that the failure to discover evidence of haploid-diploid pseudogamy does not prove that it cannot be stimulated by the use of X-rayed pollen in the same material. This phenomenon might have been revealed had more diploids appearing in the lower doses been tested; yet the same relative dosage levels were sampled as those in which IVANOV (10) found maternal diploids. Also, the occurrence of this type of apomixis might be so rare that the same experiment conducted on a much greater scale might be needed to reveal it. Nevertheless, if a technique is to be of any value

in the production of pure lines or haploids for practical or theoretical use, it must certainly yield more of the desired product for the amount of effort involved than IVANOV has obtained.

In the experiments of EAST (7), IVANOV (10), and REDINGER (19), self- and cross-contamination is admittedly a possible explanation of the origin of maternal diploid plants. This was considered by each of these workers but ruled out as unlikely. However, in none of these experiments was incontestable proof offered of the haploid-diploid method of origin. The chromosome condition of the endosperm in such cases might shed some light on the nature of the origin of the diploid embryos. Only in the work of REDINGER (19) was this approach attempted. Here diploid and tetraploid embryos in *Petunia* arose seemingly by haploid-diploid pseudogamy as a result of using pollen of *Salpiglossis sinuata*. Wherever chromosome counts were made in the endosperm of young developing seed, however, they were found to be triploid. If this triploid condition resulted in any other way except by double fertilization in amphimixis, it would certainly be an unusual case.

TABLE 2

SUMMARY OF MEASUREMENTS

Progeny no.	Pedigree	N	Length of tube		Diameter of limb	
			Mean (mm.)	Standard deviation (mm.)	Mean (mm.)	Standard deviation (mm.)
$P_{VI}119$	Inbred	3	40.69	.....	66.26	.....
$P_{IX}117a\text{-}1$	Inbred	15	65.19	0.80 ±0.15	43.79	0.96 ±0.18
$P_{IX}117a\text{-}2$	Inbred	18	64.68	1.47 ±0.25	43.05	1.80 ±0.30
$P_{IX}117a\text{-}3$	Inbred	9	66.05	1.28 ±0.30	44.18	1.54 ±0.36
$P_{IX}117b\text{-}1$	Inbred	17	70.29	0.94 ±0.16	44.66	0.90 ±0.15
$P_{IX}117b\text{-}2$	Inbred	20	68.19	0.69 ±0.11	44.76	1.32 ±0.21
$P_{IX}117$(mean)	Inbred	79	66.88	1.036	44.09	1.304
$P_{I}116\text{-}5$	F_1 (single clone)	41	54.31	0.57 ±0.06	62.42	1.67 ±0.18
$P_{II}A4$	F_2	84	54.45	3.87 ±0.30	59.51	4.28 ±0.33
$P_{III}N193$	X_2	11	54.78	2.72 ±0.58	50.09	2.07 ±0.44
$P_{III}P103$	X_2	23	60.21	5.22 ±0.77	47.09	3.35 ±0.49
$P_{III}R122$*	X_2	11	61.91	3.39 ±0.72	55.21	2.43 ±0.52
$P_{III}R124$*	X_2	18	61.98	2.40 ±0.40	57.44	2.99 ±0.50
$P_{III}R153$	X_2	15	58.01	3.89 ±0.71	49.36	2.21 ±0.40
Mean	X_2	78	.....	3.52	.....	2.61

*$P_{III}R122$ and $P_{III}R124$ both from selfings of single X_4 plant.

X-RAY–INDUCED CHROMOSOMAL ABERRATIONS

As a byproduct of the present experiment, the cytologically aberrant plants were investigated. From table 1 it can be seen that a total of thirty-one aberrants was detected from examination of root tips. By means of this method only certain types of aberration—for instance, reduction or increase in the number of chromosomes—can be identified with certainty. Translocation might be responsible for some of the changes in length, but in general root-tip figures

would not indicate the amount of translocation or inversion induced by the X-ray treatment.

Most of the plants of the X_1 generation were transferred to the field for observations on their flower characters and for further cytological investigation. For study of the pollen mother cells, buds were fixed in Carnoy's fluid and anthers smeared in iron aceto-carmine. Diakinesis of the first meiotic division proved to be an excellent stage for determining pairing relations and relative sizes of chromosomes (figs. 1, 3, 6).

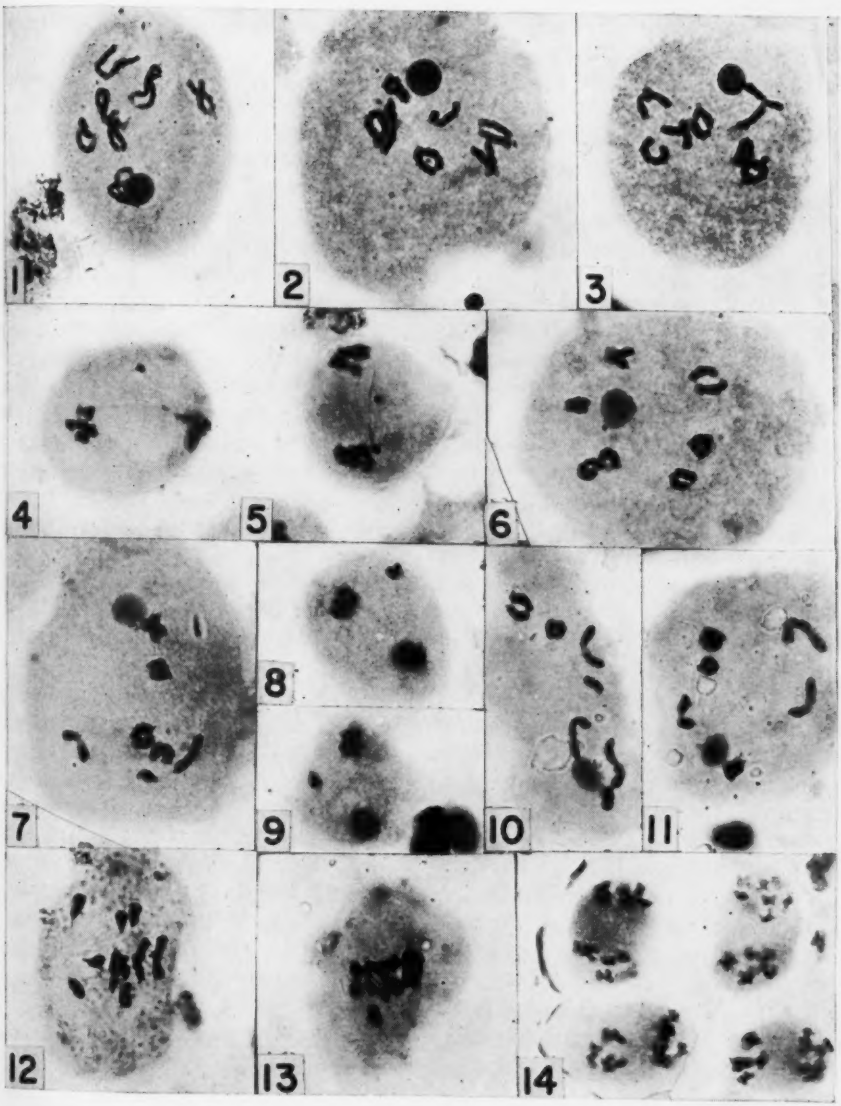
These studies indicated that in many cases the count or morphology of chromosomes in pollen mother cells differed from that in mitotic figures in root tips of the same plant. This situation was definitely established in six plants out of a sample of eighteen which were considered thoroughly enough investigated to reveal a major difference if any existed. This sample of eighteen was not by any means random; in fact, it included only plants which had previously been found aberrant from studies of root tips. It was not possible to make a complete survey of pollen mother cell divisions in all the X_1 generation, so that a random sample could not be attained. It is possible that there were other chimeras, especially of the type in which the root-tip complement was normal and that of the pollen mother cells abnormal.

The chimeras encountered in this experiment seemed to be largely of the type in which the roots as a unit deviated from the tops as a unit, similar to the case of a $2n:2n-1$ chimera in *Zea* reported by McCLINTOCK (17). Evidence of differences within the roots or within the shoots of a single plant is not nearly so abundant or so convincing. Five possible cases in a sample of thirty in root tips and one in sixteen of the pollen mother cells were found. The reservation must be added that in examining the meiotic division of pollen mother cells only the subepidermal layer of the shoots was being sampled. If a periclinal condition was involved in any of the shoots, it would obviously be overlooked. Nevertheless, there was no indication of a periclinal chimeral condition in the root tips. A few diploid-tetraploid sectorial root tips were encountered, but these were probably the result of spontaneous doubling rather than an effect of the X-ray treatment. Such diploid-tetraploid chimeras have been observed in untreated *Petunia* (4, 15).

These intra-plant differences nearly always involved the presence or absence of fragment chromosomes. In two cases the fragment chromosome was lacking in pollen mother cells; in the other four it was lacking in root tips. Thus losses were not limited to root tips, as has been recently observed in *Sorhgum* (11) and in *Paris* (5).

Presumably a fragment chromatid was lost to the cytoplasm in one of the early divisions of the embyro. According to the studies of BHADURI (1) on the embryogeny of *Petunia axillaris*, a chimera of this type could be established by a loss at the second division of the embryo. The chalazal cell resulting from the initial division of the zygote divides to produce two cells, one of which gives rise to the cotyledons and apical meristem, the other to the hypocotyl and the meristem of the radicle. Thus the postulated disturbance would need to be removed by only two mitoses from the time of exposure to X-rays in order to be effective in producing a root-shoot chimera.

The readily distinguished fragment chromosomes seemed to pair rather con-



FIGS. 1–14.— Stages of first meiotic division of pollen mother cell. Fig. 1, nucleolus-satellite relation at early diakinesis in normal ($2n = 14$). Fig. 2, diakinesis in $P_{II}L1–22$ ($2n = 13 + f$). Fig. 3, diakinesis in $P_{II}J1–2$ ($2n = 14 + f$). Figs. 4, 5, anaphase figures of $P_{II}L30–9$ (inversion heterozygote). Fig. 6, diakinesis in $P_{II}L30–12$ (translocation heterozygote). Note nucleolus-satellite relation and separation of satellites from main body of chromosome. Figs. 7–9, $P_{II}N9–1$ ($2n = 13$); fig. 7, diakinesis; figs. 8, 9, telophase. Figs. 10–14, $P_{II}L28–14$ ($2n = 13$); figs. 10, 11, diakinesis; figs. 12, 13, metaphase; fig. 14, telophase figures.

sistently with their normal partners (fig. 2), although the frequency of chiasmata was reduced—as might be expected.

Meiotic studies revealed one inversion and several reciprocal translocations. The single inversion must have been rather large, since 56 per cent of 147 figures examined in anaphase of the first division showed an inversion bridge with accompanying fragment. Nothing unusual was seen in the meiotic behavior of the translocations. One was found to involve the satellited chromosome; another (fig. 6) was characterized by a very high frequency of quadrivalent formation. All of the foregoing types of aberration have been recovered frequently from X-rayed plant material (9).

Monosomics may also be induced by X-ray treatment, but this treatment has not ordinarily been considered a technique for producing them, especially in diploid plants. It is assumed that in the ordinary sense *Petunia* is a diploid, its haploid number, seven, being the lowest yet found in the Solanaceae. Considerable attention was therefore paid to three monosomics which appeared in the X_1 generation.

One of these, plant $P_{II}N9$–1, appeared in a family which could be traced to pollen treated with 30,000 r. Earlier observations on the root tips of this plant had revealed the presence of a chromosome fragment in addition to thirteen normal chromosomes. This observation was verified by four separate collections of root tips. On the other hand, three preparations of pollen mother cells from this plant showed only thirteen chromosomes, none of which appeared abnormally short. As a matter of fact, the chimeral condition of this plant was not realized until long after the growing season. The preserved material was limited but was sufficient to establish the pairing relations in its thirteen chromosomes.

Figure 7 illustrates a typical diakinesis figure of this monosomic. The odd chromosome remained unpaired in by far the greater number of figures (87 per cent of twenty-four analyzable ones). In fact, its attachment with a rod bivalent in three figures could not be interpreted as the effect of chiasmata with any degree of certainty. At anaphase of the first division the odd chromosome tended to lag on the equatorial plate (figs. 8, 9). This lagging occurred in 71 per cent of the thirty-eight telophase figures examined. Flowers of this plant set seed by selfing, but no studies have yet been made of its progeny.

Another monosomic, $P_{II}N9$–9, was recovered from the same population. No meiotic studies could be made, since this plant died before flowering.

The remaining monosomic, $P_{II}L28$–14, was a product of pollen receiving 25,000 r. In this case some fifteen separate observations on root tips and pollen mother cells affirmed the count of thirteen chromosomes. This plant as a seedling had deformed leaves, but in later development it produced small leaves of normal shape. Flowers were considerably smaller than the smallest segregates in the F_2 of the original cross. In fact, the whole plant could be well described as diminutive. It was nevertheless healthy and required no special cultural care.

Satisfactory preparations of meiotic divisions were difficult to obtain in the case of this monosomic. Those which could be relied upon showed a behavior of the odd chromosome quite different from that of the previous case. In fifteen out of twenty-five clearly defined diakinesis figures the odd chromosome was associated in a trivalent configuration. Although this is a relatively small num-

ber, the pairing association was also observed in the majority of figures in other preparations. Typical meiotic behavior is demonstrated in figures 10–14. Since it has not been possible to distinguish morphologically between the seven *Petunia* bivalents at diakinesis, it was uncertain whether the odd chromosome was always associated with the same bivalent, yet it was never attached to the satellited bivalent. Furthermore, the behavior is so remarkably regular for a monosomic that it is doubtful whether more than one bivalent was concerned.

This high frequency of pairing was reflected in the more regular performance of the odd chromosome at anaphase. It lagged behind the other chromosomes in no more than 2 per cent of a great number of figures. This estimate was confirmed by the frequency of tetrad microspore groups which included micronuclei.

The unusual pairing behavior of this odd chromosome is attributed to genetic homology between it and the chromosomes to which it is so strongly attracted —according to the accepted explanation of chromosome pairing. As to the origin of the homologous section of this chromosome, two possibilities exist: either it is the result of a translocation effected by the X-rays or it is a duplication normally present in the haploid complement of *Petunia*. These alternatives are diagrammed in figure 15.

In the case of a naturally occurring duplication, the X-ray treatment would in some way have prevented the chromosome bearing this duplication (fig. 15 *du*) from migrating at anaphase to the sperm nucleus which functioned in fertilizing the egg nucleus. An inactivation of the centromere by an effective ionization might account for this misbehavior. Other possible ways in which this effect might have been produced are suggested in the study by SWANSON (**22**) of X-ray effects on chromosome behavior in the division of the generative nucleus in pollen tubes of *Tradescantia*. The "odd" chromosome (the one bearing the duplication) would thus enter the zygote of the monosomic from the female gamete. It would pair as an end member of the trivalent in meiosis of the monosomic.

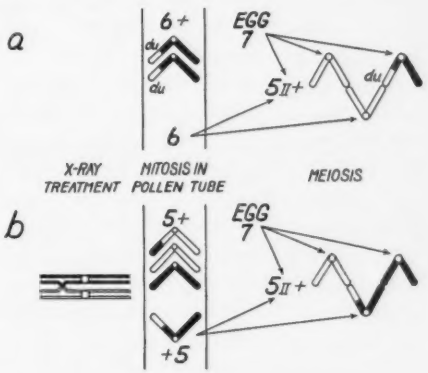


FIG. 15.—Diagrams of hypothetical origins of odd chromosome in monosomic, $P_{II}L28$–14: *a*, case of naturally occurring duplication (*du*, segment which is duplicated); *b*, case of X-ray-induced duplication.

If the homologous section could be related to the X-ray treatment, as illustrated in alternative *b* of figure 15, the radiation would be required to account for the lagging of chromatids as well as a translocation between one of the lagging chromatids and another chromatid of the six remaining chromosomes. The spreading effect of a single ionization might be held responsible if all the regions concerned were sufficiently close at the time of treatment, but it is certainly much more likely that two independent ionizations were involved. According to this alternative the chromosome with a duplication would be contributed by the sperm and in the monosomic it would

pair as the middle member of the trivalent.

In the lack of conclusive evidence, neither hypothesis can be proved or disproved; however, two points might merit attention at this point. First is the double task assigned to X-rays in the hypothesis that the radiation is responsible for the homologous section of the odd chromosome. The probability of such an occurrence is remote in view of the relative frequency of induced aberration observed in this experiment. Thus at the dose of X-rays which produced this deficient gamete, only 11 per cent of the X_1 generation showed gross chromosomal aberrations. Furthermore, in only two cases were plants found which showed two independent aberrations. In the light of these facts it does seem rather unlikely that two independent ionizations would have affected the same chromosome. Both effects might be attributed to one ionization, but this is also a rather remote possibility.

In regard to the explanation based on a naturally occurring duplication, it may seem somewhat extraordinary to postulate internal polyploidy in the complement of a plant which has the lowest chromosome count of a relatively well explored family, yet this same condition has been demonstrated by CATCHESIDE (3) in an *Oenothera* haploid. Then, too, evidence from other sources suggests the existence of duplicated regions in the *Petunia* complement. In the progeny of triploids and trisomics in *Petunia*, LEVAN (15) and, more recently, the writer have observed the presence of fragment chromosomes. Possibly these shortened chromosomes are the products of pairing in an allosyndetic sense. If internal duplications exist, competition for pairing might lead to pairing of what are ordinarily considered non-homologous chromosomes. It is conceivable that chromosomes thus paired might be displaced with respect to their ends and centromeres, so that crossovers in the paired region would lead to shortened chromosomes (fragment chromosomes), lengthened chromosomes, and possibly even dicentric and acentric chromosomes.

Other phenomena which indicate internal polyploidy are known in *Petunia*. According to unpublished findings of the writer, the effects of chromosomal unbalance on phenotype, viability, and fertility are lacking to a surprising extent. Here, and in the work of LEVAN (16), seedlings from triploid by diploid crosses exhibited chromosome numbers ranging continuously from fourteen to twenty. Even the most unbalanced of these were fairly vigorous, deviated little from the diploid in phenotype, and yielded considerable viable seed. Such results could hardly be expected in a plant which is diploid in the strict sense. Furthermore, several workers have experienced relative difficulty in obtaining cases of well-defined disomic Mendelian segregation in *Petunia*. Internal duplications and the consequent presence of genes in quadruplicate could also account for these findings. The tolerance of *Petunia* pollen to relatively high X-ray doses might also be an indication of internal polyploidy.

Neither of these points per se constitutes proof, but together they do intimate that the more likely explanation of the unusual pairing in this monosomic is to be found in naturally occurring duplications.

Summary

1. Pollen of diploid *Petunia* was subjected to X-ray doses varying from 10,000 to 100,000 r and was subsequently applied to stigmas of untreated plants. Pollen which had been exposed to doses

as high as 50,000 r functioned to produce viable seed. The half-kill dose for the number of plants produced per flowers pollinated was approximately 9,000 r.

2. Techniques were applied to the X_1 and X_2 generations in order to reveal reduced pseudogamy or pseudogamous development of diploid individuals from eggs in which a doubling of the chromosome number had occurred. No evidence of either phenomenon was observed.

3. Thirty-three plants produced by X-rayed pollen were found to have gross chromosomal deviations, including inversion, translocation, duplication, and deficiency. One-third were chimeras in which the chromosome condition of pollen mother cells differed, usually in the presence or absence of a fragment chromosome, from that of the roots. Loss of the fragment in the earliest division of the embryo presumably accounts for these differences.

4. Two monosomics were obtained. In one the odd chromosome nearly always appeared as a univalent, which usually failed to be included in either telophase nucleus in the first division and appeared as a micronucleus in later stages. In the other the odd chromosome was associated in a trivalent in the majority of figures and never lagged at anaphase of the first or second divisions. This unusual pairing is attributed either to a duplication present in the normal complement of *Petunia* or to a translocation induced by the X-ray treatment. Reasons are presented which suggest that the former hypothesis is the more likely one.

The writer gratefully acknowledges the guidance of the late Dr. E. M. EAST in the early work on this problem. Acknowledgment is also made to the Huntingdon Memorial Hospital of Boston for the use of X-ray equipment, and to Dr. MARGARET FERGUSON, who kindly furnished seed of inbred and hybrid lines of *Petunia*.

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ANALYSIS OF VARIANCE AND COVARIANCE OF CHROMOSOMAL ASSOCIATION AND BEHAVIOR DURING MEIOSIS IN CLONES OF DACTYLIS GLOMERATA[1]

W. M. MYERS[2]

Introduction

In general, autopolyploids are irregular in meiosis, the irregularities resulting in decreased fertility and in the production of aneuploid progeny. It has been shown by MÜNTZING (10, 12) and MYERS and HILL (16, 17) that *Dactylis glomerata* L., which behaves cytogenetically as an autotetraploid (x = 7), is not an exception to the general rule. The latter workers (17) found statistically significant differences among plants of this species in frequency of quadrivalents at diakinesis, unpaired chromosomes at metaphase I, lagging and dividing chromosomes at anaphase I, and micronuclei in the quartets. The occurrence of differences among plants in degree of meiotic irregularity suggests the possibility of producing strains with greater regularity of meiosis than the average of the species. Such strains should, consequently, be more stable in chromosome number and more fertile than unselected material. For an evaluation of this possibility, more knowledge is required regarding the extent and nature of variation in the various features of meiosis which have been investigated.

Material and methods

Nine clones of *D. glomerata* were selected as representative of the range in degree of meiotic irregularity found among the twenty studied previously by MYERS and HILL (17). These clones were planted in the field in rows distributed at random in three replications. Microsporocyte material was collected and fixed from one plant in each replication of six clones in 1940 and from all nine clones in 1941. The material was fixed in acetic-alcohol and stored at low temperature in the fixing solution until studied. All data were collected from fresh aceto-carmine smear slides.

For the analysis of variance involving data for 2 years, the procedure recommended by COCHRAN (1) for experiments with perennial crops has been used. For the analysis of covariance, SNEDECOR'S (22) outline was followed.

Experimental results

DIAKINESIS.—For each replication of the clones, data on quadrivalent frequency were collected from an average of 38 diakinesis sporocytes in 1940 and 24 sporocytes in 1941. In 1941, the chiasma frequency, expressed as half-chiasmata per chromosome, was determined from the same diakinesis sporocytes. These data are summarized in table 1, with the six clones arranged in ascending order on the basis of average number of quadrivalents per sporocyte for the 2 years. The averages ranged from 3.1 to 4.2 for the six clones.

The mean numbers of quadrivalents found in the three plants studied only in 1941 were within the range of the six plants studied in both years. In addition to quadrivalents and bivalents, there were found at diakinesis in these plants some sporocytes with univalents and others with a univalent plus a trivalent. The frequency of the latter type was low, the number of trivalents per sporocyte

[1] Contribution no. 45 of the U.S. Regional Pasture Research Laboratory, Division of Forage Crops and Diseases, Bureau of Plant Industry, Agricultural Research Administration, U.S. Department of Agriculture, State College, Pennsylvania, in co-operation with the northeastern states.

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varying from 0 to 0.09 (2 trivalents in 23 sporocytes) among the replicates of the nine clones. Therefore, combining the trivalents with the quadrivalents to give multivalent frequency would not change significantly the relationship among plants that is shown by the average number of quadrivalents.

quency in 1941 as in 1940, while the means for the remaining three clones showed greater differences between years.

The low mean square for replications suggested that this character was not greatly affected by the soil variations involved in this experiment. This is indicated further by a comparison of the mean

TABLE 1

FREQUENCY OF HALF-CHIASMATA PER CHROMOSOME AND QUADRIVALENTS PER SPOROCYTE AT DIAKINESIS IN NINE CLONES OF DACTYLIS GLOMERATA AND INTRA-PLANT CORRELATIONS OF CHIASMA FREQUENCY WITH QUADRIVALENT FREQUENCY IN 1941

CLONE NO.	HALF-CHIASMATA PER CHROMOSOME IN 1941 IN				QUADRIVALENTS PER SPOROCYTE IN			r*	D/F WITHIN PLANTS
	II	IV	Difference	Total	1940	1941	Average		
OG 6(12)	1.60	1.84	0.24	1.69	3.0	3.2	3.1	+0.559†	72
OG 48(28)	1.82	1.90	0.08	1.86	3.9	3.0	3.4	+0.126	73
OG 7(20)	1.64	1.86	0.22	1.73	4.0	3.5	3.8	+0.484†	44
OG 42(5)	1.87	1.92	0.05	1.90	3.8	4.3	4.0	+0.113	57
OG 48(92)	1.74	1.89	0.15	1.82	4.1	4.0	4.0	+0.308†	87
OG 2(11)	1.80	1.91	0.11	1.87	4.1	4.2	4.2	+0.312†	97
OG 48(48)	1.71	1.86	0.15	1.77	.....	3.0	.....	+0.304†	70
OG 48(47)	1.75	1.91	0.16	1.82	.....	3.1	.....	+0.228	72
OG 2(3)	1.71	1.85	0.14	1.78	.....	3.5	.....	+0.470†	53

* Correlation coefficient between total number of half-chiasmata per chromosome and number of quadrivalents per sporocyte among sporocytes within plants of clones in 1941.

† Exceeds value of r for P of 0.01 according to FISHER'S (5) table VA.

The analysis of variance of quadrivalent frequency in the six clones with data for 2 years (table 2) showed that the variances for clones and for the interaction of clones × years were statistically significant. The significant interaction of clones × years indicates that some factor or factors of the environment had a measurable effect upon quadrivalent frequency and that the effects of those factors were not the same for the different clones. The variance for clones was not significantly greater than the variance for clones × years, and the variance for years was not significantly greater than that for error (b). From table 1 it may be seen that three of the clones, that is, OG 6(12), OG 48(92), and OG 2(11), had nearly the same quadrivalent frequency in 1941 as in 1940, while the means for the remaining three clones showed greater differences between years.

The low mean square for replications suggested that this character was not greatly affected by the soil variations involved in this experiment. This is indicated further by a comparison of the mean square for error with the mean square within plants (that is, within replicates of each clone) obtained in 1941 (table 3). As pointed out by IMMER (7), MYERS (14), and others, the error mean square is composed of the mean sampling variance (in this case the variance of the mean within replicates) plus the true variance between clones within replications. In this experiment the data on quadrivalent frequency per plant were based on an average of 24.48 sporocytes in 1941. Therefore the mean variance within plants was $1.6516 \div 24.48 = 0.0675$. Actually this value is slightly higher than the error mean square of 0.0416 (the estimated true variance between clonal rows within replications is a negative quantity), indicating that the true variance must have

been very low. Since this variance is nearly zero, it would seem appropriate to accept variance within plants as a fairly reliable estimate of error for comparing different plants or clones with regard to quadrivalent frequency.

The number of half-chiasmata per chromosome (table 1) ranged from 1.69 to 1.90 for the nine clones in 1941. Data on this character were not collected in 1940. An analysis of variance (table 3) indicated that the differences among clones were highly significant. The uncontrolled variation in this character was low; the standard error of a single determination (average of 24 sporocytes per replication) was only 0.03 (table 3). Furthermore, the mean square for replications was lower than that for error, indicating little or no effect of soil heterogeneity upon chiasma frequency in this experiment. This conclusion is supported by the comparison of the error mean square with the variance within plants. The variance of the mean within plants was 0.0128 ÷ 24.48 = 0.0005. Since the error mean square was 0.0009, the estimated true variance between clonal rows was only 0.0004. The value of F for comparing the error mean square with the

TABLE 2

ANALYSES OF VARIANCE OF FEATURES OF CHROMOSOMAL ASSOCIATION AND BEHAVIOR DURING MEIOSIS IN SIX CLONES OF DACTYLIS GLOMERATA FROM WHICH DATA WERE OBTAINED FOR 1940 AND 1941

Variance due to	D/F	No. of quadrivalents		Percentage of sporocytes				Percentage of quartets with micronuclei	
				With univalents at MI		With laggards at AI			
		Mean square	F	Mean square	F	Mean square	F	Mean square	F
Clones	5	0.9466	18.94†	227.	23.16†	909.	18.92†	1364.	34.34†
Replications	2	0.0448	.....	112.	11.44†	20.	.....	76.	1.91
Error (a)	10	0.0500	.....	10.	.....	48.	.....	40.	.....
Years	1	0.1202	3.02	206.	32.41†	8.	.....	511.	22.79†
Clones×years	5	0.3412	8.58†	78.	12.33†	52.	5.54*	203.	9.08†
Replications×years	2	0.0607	.....	21.	3.26	2.	.....	126.	5.63*
Error (b)	10	0.0398	.....	6.	.....	9.	.....	22.	.....

*Exceeds F for P of 0.05.
†Exceeds F for P of 0.01.

TABLE 3

ANALYSIS OF VARIANCE BETWEEN AND WITHIN PLANTS OF FREQUENCY OF HALF-CHIASMATA PER CHROMOSOME AND OF QUADRIVALENTS PER SPOROCYTE IN NINE CLONES OF DACTYLIS GLOMERATA IN 1941

Variance due to	Half-chiasmata per chromosome			Quadrivalents per sporocyte		
	D/F	Mean square	F	D/F	Mean square	F
Clones	8	0.0141	15.22*	8	0.7464	17.95*
Replications	2	0.0002	.....	2	0.0146	.....
Error	16	0.0009	.....	16	0.0416	.....
Within plants† of clones	634	0.0128	.....	634	1.6516	.....

*Exceeds F for P of 0.01.

†Calculated by averaging mean squares for individual plants of each clone. When calculated by pooling sums of squares and degrees of freedom for within plants, the mean squares were 0.0125 and 1.6427, respectively. The slight discrepancy arose from the variable numbers of sporocytes recorded for the different clones.

variance of the mean within plants was 1.800, which gives P of less than 0.05, suggesting that variance within plants may not furnish a reliable estimate of error for comparing different plants.

The data on chiasma frequency were recorded separately for the chromosomes associated as bivalents and as quadrivalents (table 1). In all clones the number of half-chiasmata per chromosome was higher in the chromosomes of the quadrivalents than in those of the bivalents, the differences ranging from 0.05 to 0.24 among the nine clones. The results suggest that within sporocytes chiasma frequency was a limiting factor in the formation of quadrivalents, at least in some of the clones. The differences were greatest in those clones with a low chiasma frequency and least in the clones with a high chiasma frequency. This relationship would be expected on the assumption that the differences resulted from the limitation of chiasmata number upon quadrivalent formation.

To test the importance of chiasma frequency in determining variations in quadrivalent frequency among sporocytes within plants of clones, correlation coefficients were calculated. To obtain the correlation within plants (replicates) of the same clones, the sums of products and the mean squares were calculated separately for each plant, the values for the three plants of the clone were added, and r was determined from the pooled data. Of the nine correlation coefficients (table 1) three were not significant, whereas six gave P values of less than 0.01. In all three of the clones with nonsignificant coefficients, the differences in half-chiasmata between the chromosomes of the bivalents and of the quadrivalents were among the lowest obtained. In all clones the correlation coefficients were relatively low, the highest value being 0.559. In other words, not more than 31 per cent of the squared variation in quadrivalent frequency among sporocytes within plants can be attributed to variation in numbers of half-chiasmata. The results suggest that other factors, such as chance location of the chiasmata, variation among sporocytes in the relative distances between homologues at the time of initiation of prophase, and timing balance during prophase play important roles in conditioning differences among sporocytes in numbers of quadrivalents.

UNIVALENTS AT METAPHASE I AND LAGGING AND DIVIDING CHROMOSOMES AT ANAPHASE I.—An average of 160 metaphase I sporocytes from each replication of each clone in 1940 and of 182 sporocytes in 1941 were examined. Three of the clones, that is, OG 2(3), OG 6(12), and OG 7(20), were high in percentage of sporocytes with one or more univalents (table 4). On the other hand, the amount of asynapsis found in OG 48(28) and OG 48(47) was low; in fact, the averages for these clones were within the range found among normal plants of diploid *Lolium perenne* (13). The remaining four plants ranged between these extremes. In the analysis of variance of the data on percentage of sporocytes with univalents (table 2) the mean squares for clones and for replications were significantly larger than that for error (a), while the mean squares for years and for the interaction of clones × years significantly exceeded mean square for error (b). Apparently this character is affected both by environmental fluctuations from year to year and by heterogeneity in the field. Some question may be raised regarding the legitimacy of using the analysis of variance for percentage data covering the range found in these experiments. In this case, however, the magnitude of the differences was so great as to leave

little question of their statistical significance. The rank of the clones in number of univalents per 100 sporocytes was the same as in percentage of sporocytes with univalents. The analysis of variance of these data yielded results very similar to those obtained for percentage of sporocytes with univalents.

parison of mean square for the interaction of clones × years with mean square for error (b), F was greater than F for P of 0.05. Since percentage data covering a considerable range were involved in the analysis, this difference may be of doubtful significance. The differences among replications and between years were not

TABLE 4

PERCENTAGE OF SPOROCYTES WITH UNIVALENTS AT METAPHASE I, WITH LAGGING AND DIVIDING CHROMOSOMES AT ANAPHASE I, AND WITH MICRONUCLEI IN THE QUARTETS IN NINE CLONES OF DACTYLIS GLOMERATA

Clone no.	Percentage of sporocytes								
	With univalents at MI in			With laggards at AI in			With micronuclei in the quartets		
	1940	1941	Average	1940	1941	Average	1940	1941	Average
OG 6(12)	12	29	20	36	36	36	32	56	44
OG 48(28)	3	3	3	4	2	3	4	3	4
OG 7(20)	14	9	12	28	19	24	35	26	30
OG 42(5)	4	9	6	8	13	10	11	18	14
OG 48(92)	6	11	8	8	16	12	6	13	10
OG 2(11)	4	9	6	5	9	7	5	21	13
OG 48(48)	.....	5	.....	.....	7	.....	.....	4	.....
OG 48(47)	.....	3	.....	.....	2	.....	.....	2	.....
OG 2(3)	.....	24	.....	.....	29	.....	.....	28	.....

The data for each clone on frequency of lagging and dividing univalents at anaphase I were obtained from an average of 108 sporocytes per replication in 1940 and of 95 sporocytes in 1941. The clones occupied approximately the same order with regard to percentage of anaphase I sporocytes with laggards (table 4) and number of laggards per 100 sporocytes as they did with regard to metaphase I univalents. Also in the frequency of laggards at anaphase I, the two clones OG 48(28) and OG 48(47) were only slightly less regular than the average of the normal plants of *Lolium perenne* (13).

In the analysis of variance of percentage of anaphase I sporocytes, the results indicated highly significant differences among plants (table 2). Also, for com-

significant. The mean squares for errors (a) and (b) were higher in the analysis of variance of anaphase I laggards than of metaphase I univalents, although the means of these characters were not greatly different. It is possible that the greater random variation found in anaphase I laggards may have arisen from the difficulty of determining accurately in certain instances whether chromosomes were lagging and starting to divide equationally. The analysis of variance of number of laggards per 100 anaphase I sporocytes likewise showed highly significant differences among plants. In this analysis, the value of P for comparing mean square for the interaction of clones × years with mean square for error (b) was considerably less than 0.01, indicat-

ing that the interaction was statistically significant.

DICENTRIC CHROMATID BRIDGES AND ACENTRIC FRAGMENTS AT ANAPHASE I.—Dicentric chromatid bridges and acentric fragments were observed in some anaphase I sporocytes in each of the nine clones. For the six clones with data for 2 years, sporocytes with bridges and fragments ranged from 2 to 8 per cent. These differences were found to be statistically significant, suggesting that inversions of different lengths occurred in the six clones or that there were more inverted segments in some clones than in others. There was no reason to suppose that such differences would not be found. Among the twenty plants studied by MYERS and HILL (17), chromatid bridges and acentric fragments were found in all but two. One of these was OG 6(12), which showed an average of 6 per cent of the anaphase I sporocytes with such configurations in the present study. Therefore, at least nineteen of the twenty clones studied are probably heterozygous for one or more inversions.

DISJUNCTION AT ANAPHASE I.—While examining the anaphase I sporocytes for laggards, the number of chromosomes moving to each pole was recorded for all sporocytes in which the chromosomes were so well spread that both groups could be counted accurately. Of the 520 sporocytes recorded, 507 had a 14–14 distribution and 13 (2.5 per cent) had a 13–15 distribution. In many other sporocytes in which only one group of chromosomes could be counted the number was almost always 14.

QUARTETS WITH MICRONUCLEI.—For determining the frequency of quartets with micronuclei, an average of 180 and 169 sporocytes per replication were examined from each clone in 1940 and 1941, respectively. Three of the clones, OG 48 (28), OG 48(47), and OG 48(48), had only 4, 2, and 4 per cent, respectively, of the sporocytes with micronuclei. Two of these, OG 48(28) and OG 48(47), were also low in incidence of unpaired chromosomes at metaphase I and laggards at anaphase I. In the analysis of variance of these data (table 2), the mean squares for clones, years, and clones × years were significantly greater (P was less than 0.01) than their appropriate error mean squares.

Quartets with two or more micronuclei occurred frequently, particularly in those clones in which a high percentage of the quartets had micronuclei. As a result, the range among clones in total number of chromosomes lost per 100 sporocytes (as measured by the number of micronuclei in the quartets) was greater than indicated by the range in percentage of quartets with micronuclei. In this regard also the clones OG 48(28), OG 48(47), and OG 48(48) were comparatively regular.

COVARIANCE OF BEHAVIORS AT DIFFERENT STAGES OF MEIOSIS.—For the analysis of covariance, replicated data collected in 1941 from the nine clones were used throughout. A summary of the analysis of covariance of half-chiasmata per chromosome and quadrivalent frequency per sporocyte is presented in table 5. The significance of covariance cannot be tested directly; instead the correlation (r) or regression (b) coefficients may be calculated (table 6) and the significance of either one tested. If one is significant, the other also is significant, and vice versa. In this paper the significance of r has been determined from FISHER's table VA (5). For the correlation of chiasma with quadrivalent frequency, r for clones was not significant but r for total exceeded that necessary for P of 0.01.

The remaining correlation coefficients (table 6) were calculated from the analysis of covariance in the same manner. In all analyses the mean squares for clones significantly exceeded mean square for error (P was less than 0.01 in all cases). quartets with micronuclei. From present knowledge of chromosome pairing and behavior during meiosis, these relationships were expected. The correlation coefficients of quadrivalent frequency with metaphase I univalents and with ana-

TABLE 5

ANALYSIS OF COVARIANCE OF HALF-CHIASMATA PER CHROMOSOME (x) AND NUMBER OF QUADRIVALENTS PER SPOROCYTE (y) USING DATA OBTAINED FROM NINE CLONES OF DACTYLIS GLOMERATA IN 1941

Variance due to	D/F	Sums of squares due to			Errors of estimate			
		Sx^2	S(xy)	Sy^2	Sums of squares	D/F	Mean square	F
Replications	2	0.0004	−0.0017	0.0291	.....	.....	.....	.....
Clones	8	0.1129	0.4248	5.9710	.....	.....	.....	.....
Error	16	0.0148	0.0040	0.6652	0.6651	15	0.0443	.....
Clones+error	24	0.1277	0.4288	6.6362	5.1964	23	.....	.....
Difference for testing adjusted clone means					4.5313	8	0.5664	12.77*

* Exceeds F for P of 0.01.

TABLE 6

CORRELATION COEFFICIENTS CALCULATED FROM INDICATED ANALYSES OF COVARIANCE

Variance due to	D/F	A with B*	A with C*	B with C*	B with D*	C with D*	D with E*
		r	r	r	r	r	r
Clones	7	0.517	−0.601†	0.053	0.078	0.969‡	0.928‡
Error	15	0.041	−0.037	−0.192	−0.252	0.363	0.176
Total	25	0.462‡	−0.576‡	0.028	0.026	0.861‡	0.758‡

* A, half-chiasmata per chromosome; B, number of quadrivalents per sporocyte; C, percentage of metaphase I sporocytes showing univalents; D, percentage of anaphase I sporocytes showing laggards; E, percentage of quartets showing micronuclei.

† Exceeds r for P of 0.10 (table VA, 5).

‡ Exceeds r for P of 0.01 (table VA, 5).

A significant negative correlation coefficient (and therefore also regression coefficient) was found between chiasma frequency and percentage of metaphase I sporocytes with univalents. Significant positive correlation coefficients occurred between metaphase I with univalents and anaphase I with laggards, and between anaphase I with laggards and phase I laggards were very small and not significantly different from zero. These results agree with the previous report by MYERS and HILL (17) of absence of correlation between these characters.

Just as important as the correlation between the various characters is the question whether there are significant differences among clones in one character

which cannot be accounted for by its regression upon another. This problem can be investigated by use of regression, calculating estimated values for the dependent variable on the basis of its regression on the independent variable and subjecting the estimated value to the analysis of variance. A more direct approach to the problem by calculation of errors of estimate from the covariance analysis has been given by SNEDECOR (**22**). The latter method was used in these investigations. In the analysis of covariance of chiasma frequency with quadrivalent frequency (table 5), it can be seen under errors of estimate that the mean square of the difference for testing the adjusted clone means was significantly greater than the error mean square (F was greater than that for P of 0.01). The results show that there were statistically significant differences among clones in quadrivalent frequency which could not be accounted for by differences in chiasma frequency.

Errors of estimate were calculated in a similar manner from each analysis of covariance and are summarized in table 7. In all but one case the differences for testing adjusted clone means were significantly greater than error, indicating that there were real differences among clones in each dependent variable which could not be accounted for by its regression on the independent variable. The single exception was in the covariance of percentage of metaphase I sporocytes having univalents with the percentage of anaphase I sporocytes having laggards. This indicates that in this investigation all significant differences among clones in frequency of laggards at anaphase I may be attributed to variations in incidence of univalents at metaphase I.

TABLE 7

ERRORS OF ESTIMATE CALCULATED FROM ANALYSIS OF COVARIANCE OF VARIOUS CHARACTERISTICS OF MEIOSIS IN NINE CLONES OF DACTYLIS GLOMERATA

VARIANCE DUE TO	D/F	A WITH C*		B WITH C*		B WITH D*		C WITH D*		D WITH E*	
		Mean square	F	Mean square	F	Mean square	F	Mean square	F	Mean square	F
Error	15	12.58	.....	9.20	.....	24.62	.....	58.41	....	36.06	.....
Difference for testing adjusted clone means	8	160.38	12.75†	257.83	28.02†	493.83	20.06†	30.55	....	803.35	22.28†

* A, half-chiasmata per chromosome; B, number of quadrivalents per sporocyte; C, percentage of metaphase I sporocytes showing univalents; D, percentage of anaphase I sporocytes showing laggards; E, percentage of quartets showing micronuclei.

† Exceeds r for P of 0.01 (table VA, **5**).

Discussion

From the standpoint of plant breeding, the significant differences in meiotic irregularity found among clones of *Dactylis glomerata* are of considerable importance, since they suggest the possibility of selecting strains with greater meiotic regularity and probably also greater fertility. Although, as MÜNTZING (**11**) and RANDOLPH (**20**) have suggested, physiological factors may be the chief cause of sterility in some autopolyploids, there seems to be little question but that meiotic irregularity also tends to decrease fertility. At least two of the nine plants re-

ported in this paper were very nearly as regular in meiosis as normally occurring plants of the diploid species *Lolium perenne* (13). Since the differences among plants in this investigation were maintained in different replications and through two seasons, it seems logical to conclude that they are heritable. Progeny tests, the results of which will be presented elsewhere, have confirmed this supposition.

There have been reported in the literature numerous examples of genetically controlled abnormalities of meiosis, especially asynapsis at diakinesis and metaphase I, but relatively few cases have been published of small heritable variations in meiotic behavior among apparently normal plants of a species. Variations in chiasma frequency among plants or clones of the same species have been reported by DARLINGTON (3) for *Fritillaria imperialis;* SAX (21) for *Secale cereale, Vicia faba,* and *Tradescantia* species; KAKHIDZE (8) for *Secale cereale;* and MYERS (13) for diploid *Lolium perenne.* In autopolyploid *Lycopersicum esculentum,* JORGENSEN, LESLEY and LESLEY, and UPCOTT (see UPCOTT, 23, for literature citations) reported different quadrivalent frequencies. GILES (6) found fewer quadrivalents in autotetraploid *Tradescantia* than had been reported previously by ANDERSON and SAX and by DARLINGTON. He (6) attributed the difference to lower chiasma frequency or to a greater degree of chromosomal differentiation. Since the data reported for *Lycopersicum* and *Tradescantia* were not collected under the same conditions, it is impossible to judge the significance of the differences obtained. Variations in quadrivalent frequency among plants of autopolyploid grasses have been found by MYERS and HILL (16, 17) for *Agropyron cristatum, Arrhenatherum elatius,* and *Dactylis glomerata* and by MYERS (15, and unpublished) for hexaploid *Phleum pratense* and induced autotetraploid *Lolium perenne.*

Less attention has been given to variations in chromosome behavior and loss in later stages of meiosis. MYERS and POWERS (18) found heritable differences in chromosome loss among plants of *Triticum aestivum,* and MYERS (13) reported variations among plants of diploid *Lolium perenne* in incidence of unpaired chromosomes at metaphase I, laggards at anaphase I, and micronuclei in the quartets. Differences in these characters also were found among plants of the polyploid grass species studied by MYERS and HILL (16, 17) and MYERS (15). The results suggest that material is available in many autopolyploids, both natural and induced, for the selection of types with greater meiotic regularity.

With this possibility in mind, there logically follows the question of which feature of meiotic irregularity is of greatest importance in conditioning instability and decreased fertility. DARLINGTON (4), KOSTOFF (9), and others have attributed the meiotic chromosomal irregularities of autopolyploids to the multivalent association of chromosomes during synapsis. As a result of this assumption there has been a tendency to judge the regularity of autopolyploids on the basis of frequency of multivalents. Although the results with *Dactylis glomerata* do not contradict the hypothesis of the basic importance of multivalent association in conditioning meiotic irregularity, they do emphasize that multivalent frequency at diakinesis and metaphase I is an inadequate criterion of the amount of irregularity during meiosis. In these investigations frequency of quadrivalents was correlated neither with frequency of asynapsis at metaphase I nor with frequency of lagging at anaphase I. Nevertheless,

the occurrence of univalents at metaphase I, which lag and divide equationally at anaphase I, apparently is the principal factor contributing to the production of aneuploid gametes in *D. glomerata*. Unequal distribution from quadrivalents seems to have been of infrequent occurrence. Only 2.5 per cent of the anaphase I sporocytes, in which laggards were not found, had other than a regular 14–14 distribution. At least part of the cases of unequal distribution probably could have resulted from random passage of metaphase I univalents to the poles.

It cannot be concluded that this behavior is typical for all or even for most autopolyploids. In fact, unequal distribution from multivalents has been reported commonly. RANDOLPH (19) found low frequencies of univalents at metaphase I and of laggards at anaphase I in autotetraploid maize, but unequal disjunction from quadrivalents occurred commonly. In autotetraploid *Lolium perenne* (unpublished data) both asynapsis and unequal disjunction occur in considerable frequency. COOPER'S (2) results with induced autopolyploid *Medicago sativa* also indicate that improper disjunction from quadrivalents may be an important source of laggards at anaphase I in some cases. It may be concluded that the feature of meiotic irregularity of greatest importance varies with the species, and that multivalent frequency alone will not always be a reliable criterion of stability and fertility of an autopolyploid.

In the present investigations both quadrivalent frequency and percentage of metaphase I sporocytes with univalents were significantly correlated with chiasma frequency; yet quadrivalent frequency was not correlated with frequency of metaphase I univalents. These seemingly anomalous results reveal an interesting relationship among these three characters. Judging from present knowledge of chromosome pairing, it is probable that numbers of quadrivalents are conditioned by two things, (*a*) number of chiasmata and (*b*) amount of chromosomal differentiation and other factors which tend to limit prophase synapsis to particular pairs, preventing multivalent association. The former should be positively and the latter negatively correlated with quadrivalent frequency. Univalents at metaphase I likewise should be affected by these two factors. Decreased multivalent association during prophase should result in more regular bivalent formation at metaphase I. The relative importance of this factor in conditioning quadrivalent frequency and asynapsis cannot be evaluated accurately with the available data. Nevertheless, the errors of estimate from the analysis of covariance indicated that there were differences among clones in quadrivalent frequency and also in metaphase I univalents which could not be attributed to differences in chiasma frequency. In instances where the factor of multivalent pairing in prophase was sufficiently important, it would condition a positive correlation between quadrivalent and metaphase I univalent frequencies. On the other hand, chiasma frequency was, as expected, positively correlated with number of quadrivalents and negatively correlated with incidence of unpaired chromosomes at metaphase I. This relationship would tend to cause a negative correlation between the latter two characters. In the material used in the present investigations, the two factors chiasma frequency and tendency for bivalent pairing in prophase were balanced in such a manner as to leave a correlation coeffi-

cient between quadrivalent and metaphase I univalent frequency which was not significantly different from zero.

The information regarding the interrelationships of frequencies of chiasmata, quadrivalents, and metaphase I univalents is useful as a guide in selecting for greater meiotic regularity in *Dactylis glomerata*. Selection for decreased quadrivalent frequency and increased chiasma frequency eventually should result in the greatest possible degree of regularity in meiosis. Selection for low quadrivalent frequency caused by low chiasma frequency would result in types with considerable asynapsis at metaphase I, and consequently with a high frequency of aneuploid gametes.

Previous studies (**16, 17**) found more laggards at anaphase I than could be accounted for by frequency of unpaired chromosomes at metaphase I. Similar results were obtained in the present investigations with five of the clones, while there were few or no differences in the other four. As suggested by MYERS and HILL (**17**), the greater incidence of laggards at anaphase I may have resulted either from their origin from some source other than metaphase I univalents or from greater difficulty in seeing all metaphase I univalents. The analysis of covariance indicated that all significant differences in anaphase I laggards were accounted for by variations in incidence of metaphase I univalents in these investigations. Therefore, the latter explanation seems more plausible.

Summary

1. Data on chromosomal association and behavior during meiosis were collected from three replications of each of six clones of *Dactylis glomerata* L. for two years, 1940 and 1941, and of three additional clones for one year, 1941. Statistically significant differences among clones were found for number of half-chiasmata per chromosome, average number of quadrivalents per sporocyte, frequency of unpaired chromosomes at metaphase I, frequency of lagging and dividing univalents at anaphase I, and frequency of micronuclei in the quartets. All characters were recorded in both years except chiasma frequency, which was recorded only in 1941. For all the characters recorded in both years the interaction of clones × years was significant, while the difference between years was significant only for frequency of univalents at metaphase I and for frequency of micronuclei in the quartets.

2. From analyses of covariance, correlation coefficients were calculated for the various characters. The correlation coefficients (r) of quadrivalent frequency with frequency of metaphase I univalents and with frequency of anaphase I laggards were not significant, while r of chiasma frequency with metaphase I univalents was negative and significant. Significant positive values of r were obtained between chiasma frequency and quadrivalent frequency, metaphase I univalents and anaphase I laggards, metaphase I univalents and micronuclei in the quartets, and anaphase I laggards and micronuclei in the quartets.

3. By means of analyses of covariance, it was found in all but one case that there were significant differences among plants in the dependent variable which could not be ascribed to its regression on the independent variable. In the exceptional case all significant differences among plants in occurrence of laggards at anaphase I could be accounted for by variations in frequency of metaphase I univalents.

4. It is suggested that in *D. glomerata* unequal distribution of chromosomes from quadrivalents is of minor importance in conditioning other meiotic irregularities. On the other hand, the presence of unpaired chromosomes at metaphase I which lag and divide longitudinally at anaphase I, tending to be left in the cytoplasm at telophase I or telophase II, appears to play a major role in that regard. Three of the nine clones had low frequencies of metaphase I univalents and consequently few laggards at anaphase I and few micronuclei in the quartets.

5. Significant differences among clones suggest the possibility of selecting lines of *D. glomerata* which would be more regular in meiosis and possibly more fertile. The relationships found among the various characters of meiosis serve as a guide for selection.

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CYTOLOGICAL STUDIES IN RELATION TO THE CLASSIFICATION OF THE GENUS CALOCHORTUS. III.

J. M. BEAL AND MARION OWNBEY

In two previous papers (1, 2) the number and morphology of the chromosomes of twenty-eight species and three varieties of the genus *Calochortus* (Liliaceae), as recognized in OWNBEY's monograph (5), have been reported. In a subsequent study of megasporogenesis and megagametophyte development in fourteen species of this genus, CAVE (3) has reported chromosome numbers for these species as determined by BURRELL (previously unpublished). The numbers of three of these species, *C. pulchellus* n = 10, *C. umbellatus* n = 10, and *C. palmeri* n = 7, had not been reported before, and one species, *C. weedii* n = 9, had been investigated only in the variety *vestus*, which showed 2n = 18 in root tips. The numbers recorded in CAVE's paper are in complete agreement with those previously reported for the species studied by BEAL (1, 2) and by NEWTON (4), except for the entity listed as *C. davidsonianus*, for which a chromosome number of n = 14 is reported. *C. davidsonianus* was considered by OWNBEY (5) to be a minor geographical variant of *C. splendens*, a species which BEAL found to possess 2n = 14 chromosomes. Both counts have been confirmed by OWNBEY (unpublished) in material of known geographical origin. Thus it appears that the cytological difference between *C. davidsonianus* and *C. splendens* is more striking than the morphological difference. Whether or not *C. davidsonianus* should be considered a full species or merely a tetraploid variety of *C. splendens* need not be decided at the present time. It is sufficient to point out that the two are morphologically, cytologically, and geographically distinguishable.

The present paper deals with three additional species and three varieties not heretofore studied, together with *C. palmeri* on which CAVE reported but for which she figured no chromosomes.

The bulbs used in these studies were secured by OWNBEY, either through his own field work or from correspondents. The geographical source of each bulb collection and the collector's name in each instance are given in a later section of this paper. In so far as has been practicable, a herbarium specimen from each collection has been preserved. Mitotic studies were carried out by BEAL according to the method described earlier (1, 2). Meiosis in pollen mother cells was investigated by OWNBEY by means of the aceto-carmine smear technique, using anthers from plants grown out of doors at Pullman, Washington.

In conformity with earlier papers, the sequence of sections, subsections, and species followed is that of OWNBEY's monograph (5).

SECTION I. EUCALOCHORTUS

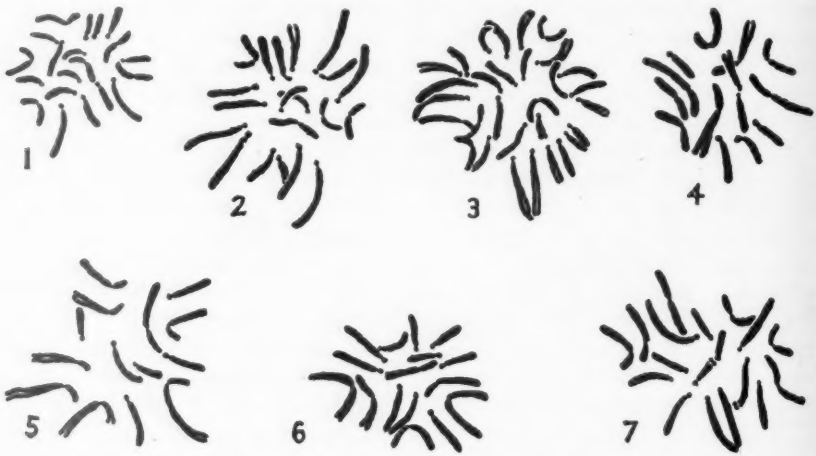
Subsection 2. Eleganti

C. elegans var. *nanus* Wood (*C. elegans* var. *oreophilus* Ownbey).—Diploid, 20 chromosomes (fig. 1); 10 bivalents at metaphase I of meiosis (not figured). The karyotype of this entity resembles more closely those of *C. lobbii* and *C. apiculatus* than it does those of *C. elegans* and *C. tolmiei*, thus suggesting that the variety *nanus* may not be so closely allied to *C. elegans* as its very similar morphology might seem to indicate. Further evidence on the genetic distinctness of these two entities has been derived from reciprocal cross pollinations between them (OWNBEY, unpublished). No hybrid seeds have been secured, although

both entities produce abundant seed under garden conditions if open-pollinated.

The material of this variety (*Ownbey & Brown 2429*) was collected in open coniferous forest along the ridge south of Gunsight Peak in the mountains west of Yreka, Siskiyou County, California. WOOD's original specimens on which this variety was based were secured in the "mountains w. of Yreka," doubtless from the same station or near it. Thus the identity of the single specimen of the original collection now preserved at the Gray Herbarium seems clearly established. It has been confused (OWNBEY, 5; and earlier investigators) with a more southern geographical variant of *C. coeruleus* (or possibly of *C. tolmiei*). The collection of OWNBEY and BROWN clearly identifies the type collection of *C. elegans* var. *nanus* with the entity recently described by OWNBEY (5) as *C. elegans* var. *oreophilus*, and the former name must therefore replace the latter as the accepted one for the entity.

Additional chromosome counts, all n = 10, have been made by OWNBEY on the following collections of this variety, all from Siskiyou County, California: *Ownbey & Meyer 2172*, from the west slope of the Siskiyou Mountains, northwest of Hornbrook; *Ownbey & Meyer 2210* and *2211*, both from the mountains southwest of Yreka; and *Ownbey & Ownbey 1748*, from the summit of the Siskiyou Mountains, 2½ miles northwest of Dry Lake Lookout. The last is the type collection of *C. elegans* var. *oreophilus*.



FIGS. 1–7.—Root-tip metaphases: fig. 1, *C. elegans* var. *nanus*, 2n = 20 (*Ownbey & Brown 2429*); fig. 2, *C. longebarbatus*, 2n = 20 (*Ownbey & Ownbey 2345*); fig. 3, *C. longebarbatus* var. *peckii*, 2n = 30 (*Ownbey & Ownbey 2360*); fig. 4, *C. flexuosus*, 2n = 14 (*Clokey 5872*); fig. 5, *C. palmeri*, 2n = 14 (*Ownbey & Ownbey 1675*); fig. 6, *C. kennedyi* var. *munzii*, 2n = 16 (*Cutler 4764*); fig. 7, *C. ambiguus*, 2n = 18 (*Leyendecker*). ×2450.

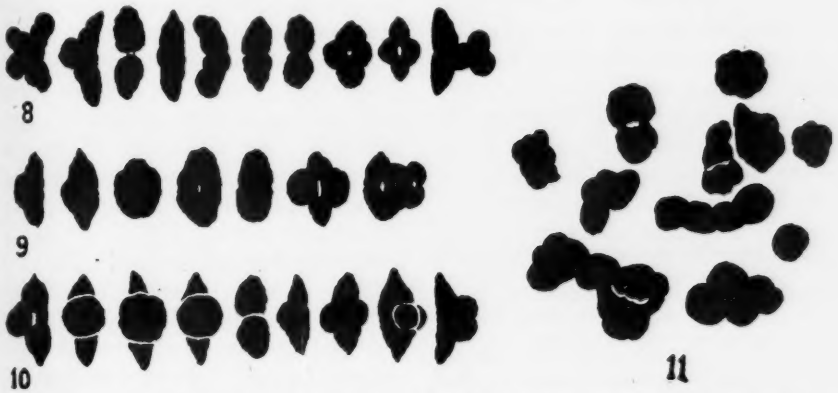
Subsection 4. Nitidi

C. longebarbatus Watson.—Diploid, 20 chromosomes (fig. 2); 10 bivalents at metaphase I of meiosis (fig. 8). The chromosomes of this species conform closely in their morphology to those of other species of the subsection Nitidi, especially to those of *C. persistens* Ownbey. Bulbs

of this latter species were secured from Mr. CARL PURDY under the name "*C. greenei*," and they were reported under that name in a former study (1) as possessing 2n = 20 chromosomes.

The present geographical distribution of *C. longebarbatus* is highly disrupted. On the basis of gross discontinuities alone, the distributional area now occupied by this species may be broken up into four or five well-separated districts. The first and largest of these covers southwestern Yakima and western Klickitat counties in Washington, and adjacent Hood River County, Oregon. This is the only area in which the species is more than occasionally met with. It was from this area also that the original material on which this species was based was collected. The record of its occurrence in Whitman County, Washington, 150 miles to the northeast, across the inhospitable semideserts of central Washington, is suspected to be in error. Intensive search at the station from which it has been reported has, so far at least, failed to disclose its occurrence there at the present time.

A second locality in which *C. longebarbatus* occurs (but here as the var. *peckii* to be described below) is in the Ochoco National Forest east of Prineville in Wheeler and Crook counties, Oregon, and a third in the same state is in the mountains about the headwaters of the Sycan River in Klamath or Lake County. Farther south it reappears in a small area in southwestern Modoc and northeastern Shasta counties, California. All these districts are separated by many miles of inhospitable habitats in which the species does not occur at the present time.



FIGS. 8–11.—Meiotic metaphase I in pollen mother cells: figs. 8–10, individual bivalents from single cell drawn separately in side view; fig. 11, polar view of metaphase plate. Fig. 8, *C. longebarbatus*, n = 10 (*Ownbey & Ownbey 2345*); fig. 9, *C. palmeri*, n = 7 (*Ownbey & Ownbey 1675*); fig. 10, *C. ambiguus*, n = 9 (*Leyendecker*); fig. 11, *C. longebarbatus* var. *peckii*, pairing irregular (*Ownbey & Ownbey 2360*). ×2700.

The material of diploid *C. longebarbatus* came from almost the opposite extremes of its known geographical range. *Ownbey & Ownbey 2345* was collected about 13 miles northwest of Goldendale, Klickitat County, Washington, a few miles east of the type locality (in "Falcon Valley") and across the canyon of the Klickitat River from it. A second collection (*Constance & Beetle 2761*) from along Satus Creek, 21 miles south of Toppenish, Yakima County, investigated by OWNBEY, likewise shows 10 bivalents at

metaphase I of meiosis. From the opposite end of the range, *Ownbey & Brown 2417* from Goose Valley, near Burney, Shasta County, California, has been investigated only by BEAL. The bulbs of this lot were collected too early in the season and were so weakened that they did not produce a sufficient number of buds for meiotic studies during their first year under cultivation. No significant differences have been noted between the karyotype of this collection and that of *Ownbey 2345*. This is rather remarkable in view of the possibility that the populations at the two stations doubtless have been separated for a very long time—perhaps for a million years.

C. longebarbatus var. *peckii* Ownbey.[1]

[1] **Calochortus longebarbatus** var. **peckii** Ownbey, var. nov., folio basilari incurvo relative longiore et angustiore basilariter rubro-purpureo; caule humiliore; floribus crateriformibus, petalis latioribus brevioribus prope glandulam abrupte flectis, cuneolo librato et limbo erecto; aliter similis speciei.—OREGON: grown from bulbs of *Ownbey & Ownbey 1800* from grassy margin of wet meadow, headwaters of Marks Creek, Ochoco National Forest, Wheeler County (TYPE, in the Herbarium of the State College of Washington); also from bulbs of *Ownbey & Ownbey 2360* from grassy places under pines, along North Fork of Crooked River, just above the mouth of Deep Creek, S. 27, T. 14 S., R. 22 E., Crook County. Named in honor of its discoverer, Professor MORTON E. PECK of Willamette University, who had collected it at the Marks Creek station (*Peck 17200*), and who directed the writer (OWNBEY) to the exact locality during the summer of 1938.

One familiar with herbarium material only might easily minimize the morphological characters which distinguish the variety *peckii* from typical *C. longebarbatus*, but when living material is examined, it may be recognized at nearly all stages of development. Before flowering, the relatively longer and narrower basal leaf, which is reddish at the base and arcuate inward, is characteristic. Later it may be recognized by its lower stature and especially by its bowl-shaped flowers. The latter are particularly striking. The petals, which are relatively broader and shorter than in the species, are sharply bent at the gland, with the claw horizontal and the limb vertical. This characteristic flower shape is found elsewhere in the genus only in the related but amply distinct *C. greenei*. Flowers of typical *C. longebarbatus* are obconic campanulate.

—Triploid, 30 chromosomes (fig. 3). The occurrence of each of the chromosome forms in triplicate suggests that this variety is an autotriploid. At metaphase I of meiosis, however, the association of the chromosomes into configurations other than trivalents reveals that this is not entirely the case. In most of the cells examined the chromosomes were formed into eleven or twelve groups (fig. 11), some of which were bivalents, some trivalents, and some quadrivalents. In other cells configurations containing apparently more than four chromosomes, and also an occasional univalent, may occur. In none of the meiotic material examined has it been possible to account for all the 30 somatic chromosomes with certainty.

Notwithstanding the great irregularities at meiosis, development proceeds normally until after the tetrad has broken apart to form the individual microspores. Thereafter maturation is arrested at various stages, until at anthesis between 80 and 90 per cent of the pollen is visibly imperfect. That only a small amount of the pollen which matures is actually functional was indicated when the anther contents were applied to covered stigmas of typical *C. longebarbatus*. This cytologically regular material (n = 10) from Klickitat County, Washington, produces an abundance of seed under usual conditions, but only a few abortive seeds, mostly without embryos, resulted when twelve flowers were pollinated with an excess of pollen from var. *peckii*. Furthermore, in the fifty-one fruits which have been allowed to develop on var. *peckii* during the last two seasons, not a single seed was formed, although an abundance of viable pollen must have reached the stigmas from the Klickitat County collection growing by its side. Similar observations were made

in the field in 1940, when not a single seed was found although many fruits were examined.

The probability that *C. longebarbatus* var. *peckii* is unable to reproduce itself by seed seems well enough established. To most species of the section Eucalochortus this would be a fatal handicap, for, although all of them—so far as is known—reproduce vegetatively through division of the bulb, this is far too slow a process in most cases to maintain the species. *C. longebarbatus*, however, is one of the two species of this section which regularly produce bulblets in the leaf axils at or below the surface of the ground. With this effective means of vegetative reproduction, *C. longebarbatus* var. *peckii* has not only maintained itself since its origin but has apparently been able to increase its numbers. If all individuals of this variety represent only one clone, as is here suggested, then its distribution is indeed of significance. At the present time it is known only from the two localities cited. These are about 18 miles apart and are now separated by an expanse of habitats in which the variety does not grow. Assuming that the original point of dispersal was halfway between the two known stations, it has been necessary for this variety to migrate at least 9 miles since its origin. If it were to move at an average rate of 1 inch per year in a straight line (an exceedingly generous estimate), it would take well over half a million years to migrate 9 miles. Perhaps in a case of this kind transport by rodents, or possibly by water (although the two stations are on different drainage systems), would be more important than mere growth. The probability remains, however, that *C. longebarbatus* var. *peckii* is very old. Considering this, the cytological and morphological uniformity of the plants from the two localities becomes truly remarkable. Is it possible that evolution has been at a standstill in this clone for 500,000 years? This should be expected, of course, since the genes are presumably in triplicate, and there is no provision for their reassortment through reduction division. Barring dominant gene mutations, the rate of phenotypic change should approximate the cube of the mutation rate of a given gene. This would impose a handicap of great evolutionary consequence on those characters governed by even the most labile of the genes. If the variety *peckii* is old, then meiotically-regular *C. longebarbatus* must be older. Although var. *peckii* appears to have remained constant since its origin, it seems unlikely that sexually-reproducing *C. longebarbatus* would have done likewise during the same period of time. It seems possible, therefore, that here in var. *peckii* are some of the genes which have been eliminated from *C. longebarbatus* in the last 500,000 years. Hybrids, if they can be obtained, and apparently they can, should be very interesting.

The resemblances between *C. longebarbatus* var. *peckii* and *C. greenei* may also be of significance in the interpretation of the phylogeny of the Eucalochorti. As Ownbey (5) has pointed out, the center of dispersal of this section appears to be coincident with the ancient Klamath land area of northern California and southwestern Oregon. This does not mean that the present-day species originated on and migrated out from the Klamath area, but that at some time the ancestor or ancestors of these species occupied this area as a refuge during an unfavorable era. *C. greenei* now occurs only within that area, but *C. longebarbatus* has a much disrupted distribution, extending from Shasta County, California, to Washington. Geographically, *C. longe-*

barbatus var. *peckii* occurs between *C. greenei* and typical *C. longebarbatus* in southern Washington and northern Oregon. Its resemblance to *C. greenei* suggests that it may have retained certain genes in common with that species which have been lost by its sexually-reproducing relative. The few collections of *C. longebarbatus* from southern Oregon and northern California are all old and are inadequate for determining whether or not they are identical with the material from farther north. Living material secured in Goose Valley, Shasta County, during the summer of 1941 (*Ownbey & Brown 2417*) should be of assistance in this connection.

Section II. Mariposa

Subsection 5. Venusti

C. flexuosus Watson.—Diploid, 14 chromosomes (fig. 4). Karyotype similar to that of *C. catalinae*, with both species showing only 4 chromosomes with subterminal centromeres, and the remaining 10 have median or submedian centromeres. The two species are evidently closely related, both cytologically and morphologically.

The material of this species was collected by Mr. Ira W. Clokey (no. 5872) on gravelly slopes and hillsides at Glendale Junction, about 50 miles northeast of Las Vegas, Clark County, Nevada.

C. palmeri Watson.—Diploid, 14 chromosomes (fig. 5); 7 bivalents at metaphase I of meiosis (fig. 9). The chromosomes of this species conform closely in morphology to those of *C. splendens*, with which it is closely allied otherwise. Both show 6 chromosomes with subterminal centromeres, and the remaining 8 have similar forms and centromere positions in the two species.

The material of *C. palmeri* (*Ownbey & Ownbey 1675*) was collected in a grassy swale along the south side of Big Bear Lake, San Bernardino Mountains, San Bernardino County, California.

Subsection 7. Nuttalliani

C. kennedyi var. *munzii* Jepson.—Diploid, 16 chromosomes (fig. 6). The karyotype of this variety is scarcely distinguishable from that of the species, of which it is perhaps no more than a color form.

Bulbs of this variety were removed from herbarium specimens collected by Dr. Hugh C. Cutler (no. 4764) on a grassy plain, 8 miles southeast of Sonoita, Santa Cruz County, Arizona. No material was available for meiotic studies.

C. ambiguus (Jones) Ownbey.—Diploid, 18 chromosomes (fig. 7); 9 bivalents at metaphase I of meiosis (fig. 10). The karyotype of this species is almost identical with that of *C. gunnisoni*, and the two species are otherwise very similar. On the basis of their very close morphological resemblance, these two species, together with a variety of *C. gunnisoni*, were placed together by Ownbey (5) to constitute the subsection Gunnisoniani. The cytological evidence now supports that derived from morphology in indicating the validity of this subsection.

The material of *C. ambiguus* was collected about 1 mile west of Cloverdale, in the southwestern corner of Hidalgo County, New Mexico, by Mr. P. J. Leyendecker. Additional material from 2 miles southeast of Sonoita, Santa Cruz County, Arizona, collected by Dr. Hugh C. Cutler, was investigated only by Beal. There appears to be little or no karyotypic difference between the two collections.

Discussion

Including the species and varieties investigated in the present study, a total of thirty-six species and six varieties of

the genus *Calochortus* have been examined cytologically. This total includes *C. davidsonianus*, *C. kennedyi* var. *munzii*, and *C. longebarbatus* var. *peckii*, which were not included among the fifty-seven species and twelve varieties of this genus accepted by OWNBEY (5). In table 1 the chromosome numbers of the species and varieties which have been investigated cytologically are summarized. In order to demonstrate the close correlation between chromosome number and systematic position existing in this genus, the sequence of sections, subsections, species, and varieties is that of OWNBEY'S monograph. In each case the name of the investigator or investigators responsible for the count is indicated in the right-hand column.

Of the twenty-one species and five varieties now recognized in the section Eucalochortus, eighteen species and three varieties have been investigated cytologically. All of these have shown 10 as the basic chromosome number. All are diploid except *C. pavonaceus* and *C. uniflorus*, which are tetraploid, and *C. longebarbatus* var. *peckii*, which is triploid.

Among the twenty-two species (including *C. davidsonianus*) and seven varieties (including *C. kennedyi* var. *munzii*) of the section Mariposa, sixteen species and two varieties have been cytologically examined. All ten species of the subsection Venusti which have been investigated show 7 as the basic chromosome number, at least in part. Eight of these species are diploid and two are tetraploid. In two species a basic chromosome number other than 7 is found in some localities. In *C. superbus*, two cytological races, one n = 6 and one n = 7, are scarcely to be distinguished on tangible morphological characters. The same is true of *C. luteus*, where there is only a cytological distinction between the race with n = 7 and that with n = 10 chromosomes. In both of these cases major cytological changes have taken place, apparently so recently that there has been no time for genetic divergence. In other words, here are two cases in which genetic isolation through gross cytological change has preceded morphological differentiation. It may be assumed that, if the two races in each instance persist long enough, such morphological differentiation may appear. Indeed, such may have been the origin of the natural groups of *Calochortus* now characterized by a distinctive chromosome number.

Like most of the species of the subsection Venusti, the single species included in the subsection Macrocarpi has a basic chromosome number of 7. In the subsection Nuttalliani, however, the three species and two varieties for which a chromosome number has been reported have a basic number of 8. *C. nuttallii* var. *aureus*, the only tetraploid of the lot, is as distinct morphologically, cytologically, and geographically as is *C. davidsonianus*. It may be well to point out here that the basic number of 8 is not constant for the entire subsection Nuttalliani (OWNBEY, unpublished).

The two species included in the subsection Gunnisoniani, section Mariposa, have a basic chromosome number of 9, with no known polyploidy. It is probably significant that these two species are more similar morphologically to the species of the subsection Nuttalliani (n = 8) than they are to species of the subsections Venusti or Macrocarpi (n = 7). They are, of course, very different from the species of the section Cyclobothra (n = 9) and of the section Eucalochortus (n = 10). If 7 is the ancestral number for the genus, as has been suggested by BEAL (1), this would indicate that these higher basic numbers have been derived

TABLE 1

REPORTED CHROMOSOME NUMBERS IN THE GENUS CALOCHORTUS, WITH SPECIES AND VARIETIES ARRANGED INTO SUBSECTIONS AND SECTIONS ACCORDING TO THEIR NATURAL AFFINITIES AS INDICATED BY MORPHOLOGICAL AND CYTOLOGICAL EVIDENCE

Section	n	2n	Investigator
Section I: Eucalochortus			
Subsection 1. Pulchelli			
C. amoenus	10*	20	Beal, Burrell
C. albus	10*	20	Beal, Burrell, Newton
C. pulchellus	10*	.....	Burrell
C. amabilis	10*	20	Beal, Newton
Subsection 2. Eleganti			
C. monophyllus (C. benthami)	10*	.....	Burrell, Newton
C. tolmiei	10*	20	Beal
C. elegans	(10)*	20	Beal
C. elegans var. selwayensis	(10)*	20	Beal
C. elegans var. nanus†	10	20	Beal and Ownbey
C. lobbii	(10)*	20	Beal
C. apiculatus	10*	20	Beal
Subsection 3. Nudi			
C. umbellatus	10*	.....	Burrell
C. uniflorus	20*	40	Beal
C. nudus	(10)*	20	Beal
Subsection 4. Nitidi			
C. longebarbatus	10	20	Beal and Ownbey
C. longebarbatus var. peckii	?	30	Beal and Ownbey
C. pavonaceus‡	20*	40	Beal
C. nitidus	(10)*	20	Beal
C. howellii	10*	20	Beal
C. lyallii	10*	20	Beal
C. persistens	(10)*	20	Beal (as C. greenei)
Section II: Mariposa			
Subsection 5. Venusti			
C. catalinae	7*	14	Beal, Newton
C. flexuosus	.....	14	Beal and Ownbey
C. palmeri	7	14	Beal and Ownbey, Burrell
C. splendens	7*	14	Beal
C. davidsonianus	14*	.....	Burrell
C. venustus	7*	14	Beal, Burrell, Newton
C. superbus	6,* 7*§	12, 14§	Beal
C. vestae	14*	28	Beal, Burrell, Newton
C. luteus	7,* 10*	14, 20, 21	Beal, Burrell, Newton
C. leichtlinii	(7)*	14	Beal
Subsection 6. Macrocarpi			
C. macrocarpus	(7)*	14	Beal
Subsection 7. Nuttalliani			
C. nuttallii	(8)*	16	Beal
C. nuttallii var. aureus	.....	32	Beal
C. kennedyi	.....	16	Beal
C. kennedyi var. munzii	.....	16	Beal and Ownbey
C. clavatus	8*	16	Beal, Burrell

* Count independently confirmed by OWNBEY (unpublished). Numbers to be inferred from the mitotic count, but previously unreported, are inclosed in parentheses.

† *C. elegans* var. *oreophilus* Ownbey, not *C. coeruleus* var. *nanus* Ownbey.

‡ Use of the name *C. douglasianus* for this entity cannot be maintained.

§ Reported as *C. luteus* var. *oculatus*, a misdetermination.

TABLE 1—*Continued*

Section	n	2n	Investigator
Subsection 8. Gunnisoniani			
C. ambiguus	9	18	Beal and Ownbey
C. gunnisoni	(9)*	18	Beal
Section III: Cyclobothra			
Subsection 9. Weediani			
C. plummerae	9*	18	Beal, Burrell, Newton
C. weedii	9*	.....	Burrell
C. weedii var. vestus	(9)*	18	Beal
Subsection 11. Barbati			
C. barbatus	(18)*	36	Beal

independently upon at least three different occasions. This hypothesis is adequately supported by geographical evidence. The section Eucalochortus is primarily northwestern in distribution, with a center in northern California. The section Cyclobothra is found only in southern California and Mexico. The two species of the subsection Gunnisoniani are limited in their distribution to the Rocky Mountains and the deserts of the southwest. In only one instance do species belonging to any two of these entities occupy the same area. In southern California, the range of *C. albus* (section Eucalochortus) includes territory occupied by species belonging to the subsection Weediani (section Cyclobothra).

All the species of the section Cyclobothra which have been examined cytologically show 9 as the basic chromosome number. There is one known case of tetraploidy. Most of the species of this group are Mexican, however, and for this reason living material has been unavailable for cytological study. It is hoped that anyone having opportunity to collect bulbs of one or more of these species will get in touch with the writers.

The data presented here show conclusively that a natural taxonomic system of classification is possible in the genus *Calochortus* which satisfies the requirements of the cytological, geographical, and morphological evidence. This system closely parallels that outlined by OWNBEY (5), wherein the species are arranged according to the maximum correlation of morphological characters into twelve subsections and three sections. Resemblance in chromosome number and karyotype morphology between species so associated is marked. Each species group, whether sectional or subsectional, is characterized by a geographical distribution differing from that of any other species group. Thus it seems clearly established that OWNBEY'S arrangement of the genus *Calochortus* into sections and subsections accurately indicates actual phylogenetic relationships.

Summary

1. Chromosome counts for the following species and varieties of *Calochortus* are reported: *C. elegans* var. *nanus*.—Diploid, 20 chromosomes; 10 bivalents at metaphase I of meiosis. *C. longebarbatus*.—Diploid, 20 chromosomes; 10 bivalents at metaphase I of meiosis. *C. longebarbatus* var. *peckii*.—Triploid, 30 chromosomes; irregular pairing at metaphase I of meiosis. *C. flexuosus*.—Diploid, 14 chromosomes. *C. palmeri*.—Diploid, 14 chromosomes; 7 bivalents at metaphase I of meiosis. *C. kennedyi* var. *munzii*.—

Diploid, 16 chromosomes. *C. ambiguus.*—Diploid, 18 chromosomes; 9 bivalents at metaphase I of meiosis. All previously reported meiotic counts have been confirmed in material of known geographical origin and certain identity. Meiotic counts have been made in the following species and varieties for which this number might be inferred from the previously-reported mitotic count: *C. elegans*, 10 bivalents; *C. elegans* var. *selwayensis*, 10 bivalents; *C. lobbii*, 10 bivalents; *C. nudus*, 10 bivalents; *C. nitidus*, 10 bivalents; *C. persistens*, 10 bivalents; *C. leichtlinii*, 7 bivalents; *C. macrocarpus*, 7 bivalents; *C. nuttallii*, 8 bivalents; *C. gunnisoni*, 9 bivalents; *C. weedii* var. *vestus*, 9 bivalents; *C. barbatus*, 18 bivalents (some irregularity).

2. *C. longebarbatus* var. *peckii*, a naturally occurring, sterile, triploid clone, is described, and its biology, relationships, and probable history are discussed in detail. The application of the name *C. elegans* var. *nanus* is established.

3. All species and varieties of the genus for which a chromosome number has been reported are arranged into subsections and sections according to the sequence of OWNBEY. The section Eucalochortus is characterized by a chromosome base number of 10, with two tetraploid species and a triploid variety. The subsection Venusti of the section Mariposa has 7 as a base number, with secondary base numbers of 6 and 10 in two species in part of their range. There are two tetraploid species in this subsection. The subsection Macrocarpi of this section likewise has 7 as a base number, with no known exceptions. All species of the subsection Nuttalliani for which a chromosome number has been reported have 8 as a base number. There is one tetraploid variety. Finally, in the subsection Gunnisoniani of the section Mariposa, 9 is the only reported base number. In the section Cyclobothra the few species and the varieties which have been investigated cytologically all show 9 as the base number, with a single known instance of tetraploidy.

4. It is concluded—on the basis of morphological, cytological, and geographical criteria—that the classification as outlined by OWNBEY accurately indicates actual phylogenetic relationships.

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MEIOSIS IN AUTOTETRAPLOID SECALE CEREALE[1]

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Introduction

Cytological studies in induced autotetraploids seem to be important for an understanding of the apparent stability and uniformity of the autotetraploid condition and of both the natural and practical consequences of its induction. While the theoretical possibilities of autotetraploidy have been determined adequately, the actual results seem to deserve more attention than they have had. The present investigation has been directed toward determining the meiotic anomalies which occur in an induced autotetraploid, *Secale cereale*.

Most of the cytological investigations on autotetraploids have been made in species which have been autotetraploid for an undetermined number of generations. Consequently, the simple unmodified autotetraploid meiosis cannot be accurately described, the meiotic process having undergone selection for many generations. Furthermore, nothing can be known in many autotetraploids of the origin of the tetraploid condition; under some circumstances the tetraploid should be "homozygous" and under others it may be "heterozygous." It is possible that polyploids which differ in this respect, or in other possible directions, would act differently at the reduction divisions. The simplest case for analysis would seem to be that of an autotetraploid which had arisen by somatic doubling within a generation or two of the time at which the observations were made.

MATERIAL AND METHODS.—The material used for these observations was autotetraploid *Secale cereale* with spring habit. It was derived directly from the diploid strain by somatic doubling, presumably during the seedling stage. The original diploid was examined many times—in connection with other work as well as with the present— and found to be in no way unusual at meiosis. All the preparations were made from material which had been fixed from 2 days to several weeks in a solution of 6 parts ethyl alcohol, 2 parts chloroform, and 1.5 parts acetic acid. This fixed material was kept in a refrigerator until used. Such treatment obscures the finer meiotic details which have been shown to be present in this material, especially the chromosome coils (3), but it has the advantage of affording large quantities of microsporocytes over a long period. The preparations were made with aceto-carmine in the usual manner. All photographs were taken with a Leitz Makam photomicrographic camera in conjunction with apochromatic objectives. Both the diploid and tetraploid rye were given the writer by Dr. KARL SAX, the latter when it was but one meiosis removed from its somatic doubling.

Experimentation

GENERAL OBSERVATIONS

The autotetraploid microsporocyte of *S. cereale* is very similar to that of other described autotetraploids—*Zea mays*, *Tradescantia virginiana*, etc. At diakinesis and metaphase the chromosomes occur as univalents, bivalents, trivalents, and quadrivalents. A metaphase which contains various chromosome associa-

[1]This study is a part of the program supported by funds obtained under Bankhead-Jones Project SRF-2-5, Division of Cereal Crops and Diseases, Bureau of Plant Industry, Agricultural Research Administration, U.S. Department of Agriculture, and the Missouri Agricultural Experiment Station, cooperating.

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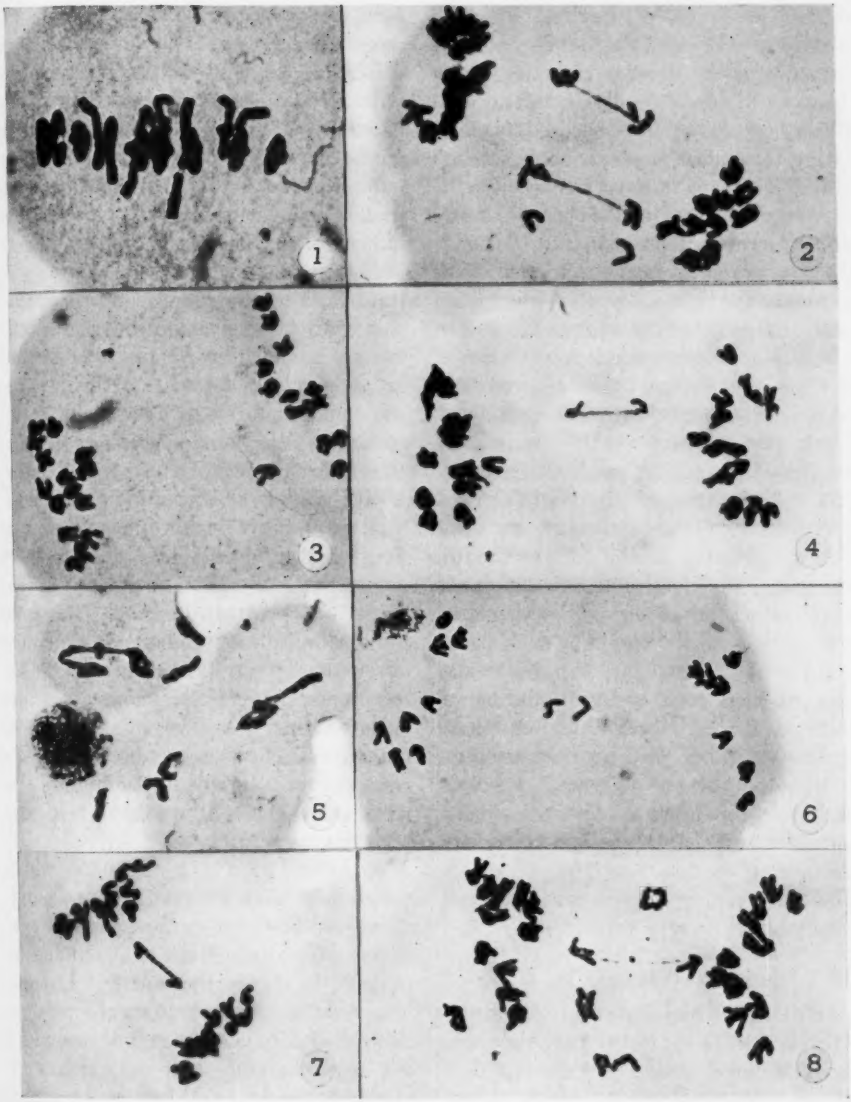
tions may be seen in figure 1. Such associations (not all present in the figure) are ring and rod bivalents, chains of three, and chains and rings of four. While these are the usual configurations, more complex and unusual types infrequently occur, that is, ring trivalents, triple arc trivalents, and interlocked quadrivalents. The univalents seem to occur in random positions in the microsporocyte when the associated chromosomes are oriented on the metaphase plate. After the associated chromosomes have disjoined and passed toward the poles, the univalents pass to the plate and split, each half passing to its nearer pole from one to several chromosome lengths behind the main group of anaphase chromosomes. Many such univalents may be seen in figures 2, 6, and 8. These photomicrographs were taken of cells which had been flattened sufficiently to bring all the chromosomes into one focus; consequently the distances between the chromosomes are exaggerated along the division axis in side views. It is therefore probable that a degree of lagging is indicated for the univalents which greatly exceeds that found in the unaltered material. At this point, early telophase, various degrees of difference become manifest in the time consumed by the univalents in reaching the plate. Some univalents split early enough to be included in the telophase nuclei, while others are left in the cytoplasm, where they form micronuclei. Stages between these extremes are frequent, and many univalents are but partially included in the telophase nuclei, the section nearest the plate being excluded by the nuclear membrane which seems to pass through the chromosome arm. At division II the process is usually normal in appearance, except for the behavior of the previously univalent chromosomes, which, having effectively but one thread, cannot split as the other chromosomes and so pass to the poles at random. Owing to the delay with which these chromosomes are oriented on the plate, various degrees of lagging now occur, and as a consequence many micronuclei are formed. These micronuclei are most clear in the quartets, where they show themselves to be small spheres, often very different in diameter and consequently far more different in volume. The pollen has irregularities in size and shape consistent with the observable meiotic anomalies and characteristic of most other autotetraploids.

These are the usual events at meiosis in the tetraploid. One exception is to be found in nonlagging univalents—univalents which do not reach the metaphase plate and split. These are included wholly in the telophase nuclei, or (rarely) are so distant from the telophase mass that they form micronuclei at interphase. It could not be determined to what extent this failure to reach the plate occurred, since once the disjunction has occurred it is impossible to decide how a single isolated chromosome reached the polar end of a cell. This is so because some chromosomes appear to reach the pole before their partners, and because in flattening the preparations the relative positions of the chromosomes are very often, if not always, changed. However, from many extreme instances in which the main body of chromosomes was in the anaphase stage and the univalents were behaving as characteristic laggards (splitting on the plate), some univalents were observed at or near the poles with their arms pointed away from the metaphase plate, a position which would seem to indicate that the chromosome had not been involved in any way in the division. It seems probable, therefore, that the anaphase laggards do not represent all the



FIGS. 1–8.—Fig. 1, metaphase plate showing characteristic chromosome associations; fig. 2, anaphase I with inversion or duplication bridges; fig. 3, anaphase I with a 15–13 distribution; fig. 4, univalent bridge at anaphase I; fig. 5, two contiguous cells, each deficient for three complete chromosomes; contiguity suggests that complete mitosis was achieved in spite of this large deficiency; fig. 6, anaphase I showing large loss of chromosomes and split laggard; fig. 7, bridge at anaphase II; fig. 8, anaphase I with three laggards and inversion or duplication bridge with fragment.

metaphase univalents but only those so placed as to be drawn into the spindle. A measure of the differences in frequency between metaphase univalents and anaphase laggards could be secured by tabulating the numbers which occur at each stage. This would be very simple for the anaphase laggards, which occur with precise structure and position; but the metaphase univalents may be concealed in the chromosome mass on the metaphase plate, behind that mass, or above it. For obvious optical reasons it is not always, perhaps not often, safe to conclude whether a suspected chromosome is loosely associated with a partner, intimately involved in a multivalent, or merely mechanically entwined, in well-flattened preparations. The validity of this decision is confirmed by the data secured in an attempt to determine this difference in frequency between anaphase laggards and metaphase univalents by such a tabulation. In many plants more anaphase laggards were counted than could be accounted for by observable metaphase univalents. These data are obviously unreliable and are not presented. It seems probable that almost all univalents reach the plate and that a small percentage of nonlagging, nonsplitting ones do occur. The relative frequencies will be dealt with later.

Univalents

One of the chief features of the autotetraploid microsporocyte is the asynapsis. The chromosomes are associated in all possible combinations which do not exceed four members (in plants which are strictly euploid). This presumably occurs because each chromosome is to some extent impeded in its attempt to associate with a partner, and thus many chromosomes fail completely to pair. The exact nature of this inability invariably to form bivalents or quadrivalents is not now clear. Several explanations are conceivable. (*a*) The spatial relationships within the nucleus are so different from those in the diploid that the homologous chromosomes have longer and more difficult paths to traverse in order to pair; the diploid may be below this threshold of difficulty. (*b*) The chromosomes may be able to pair homologously so far as spatial considerations are concerned, but some may be so situated that the attractions of homology with two other threads are so evenly balanced that the chromosome may not move effectively in response to either homologous thread, and thus at least two univalents would occur. (*c*) It is also possible that many of the associations may be so incomplete and weak that univalents split off from them before the late diakinesis or early metaphase stages are reached. (*d*) The tetraploid condition may be one in which occur various physiological unbalances affecting many intracellular processes, and thus the effect on meiosis might be a general adverse effect on synapsis, which is a common result of the application of unusual external conditions (heat, cold, and reagents).

No information is now available which would indicate how any of these possibilities could be tested or how the observations on existing material could be planned to clarify the matter. The following data were secured from a random sample of fifty autotetraploid individuals, approximately half of which were grown in the field and half in the greenhouse, so that environmental effects could be noted. A careful arrangement of the data showed no differences between the two groups, therefore they are treated as a single group. The observations were made and tabulated in the following manner. Anthers which had a

high percentage of anaphases were reduced to a very fine suspension on the slide, which was covered, heated, and then flattened to a degree which placed all the anaphase chromosomes in approximately a single focus. If carefully done, this flattening spreads the chromosomes over an area larger than usual and increases the separation between the anaphase groups. The univalent chromosomes, if there are any, are left clear and unmistakable in the middle of the cell. In this section the term univalents will indicate univalents observed or counted at anaphase. It will be clear that these univalents are the only ones which can be counted with accuracy in usable frequencies. At diakinesis, and especially at metaphase, the univalents can occur in positions which cannot reliably be determined to signify association or asynapsis. The anaphase univalents seem to constitute most, if not practically all, of the univalents in the material. Those not passing to the plate will be considered later.

In the 5000 anaphase cells examined—100 cells from each of fifty plants chosen at random—the average number of univalents was 0.72 per cell. The range of the univalent frequency was relatively wide, extending from 0 to 8 univalents, an interval which includes approximately 29 per cent of all the chromosomes in the euploid cells. These fifty plants were made up of three groups which had different somatic chromosome numbers. Six plants had 29 chromosomes, four had 27, and forty had 28. These groups were kept separate, so that the effect of chromosome number on synapsis might be determined. The aneuploidy presumably might increase the chromosome unbalance and so affect the pairing. The six plants with 29 chromosomes had an average of 0.99 univalents per cell, and the four plants with 27 chromosomes had 0.70 univalents per cell. The euploid plants, those with 28 chromosomes, had 0.68 univalents per cell. The data are presented in table 1.

If the 28- and 29-chromosome groups are compared, it will be seen that the differences between them seem relatively large. Such a comparison was made on the basis least favorable for showing a difference. The groups were compared, not on the basis of the total number of univalents but with respect to the total number of cells with univalents. Table 1 indicates that the 29-chromosome group had more cells with higher univalent numbers, a fact which would increase the difference if it were based on total univalent number. The difference between the 28- and 29-chromosome groups, computed on the basis of cells with univalents, is 16 per cent and $X^2 = ca.\ 30$. It seems clear that the increase in asynapsis which occurs in individuals with $4n + 1$ chromosomes, when compared with that in 4n individuals, is greater than can be accounted for by assuming that the extra chromosome fails to pair. It seems clear that the extra chromosome in these six individuals introduced an unbalanced condition which impeded the pairing or induced precocious separation of the chromosomes. The data seem to indicate that the addition to the euploid number was more serious than subtraction from it, for the magnitude of difference between the 28- and 27-chromosome groups is not nearly so great. The difference between these two groups is 5.7 per cent and $X^2 = ca.\ 3.4$, a difference which may be presumed to be without significance in this material. These values indicate that in this autotetraploid the additional asynapsis induced by increasing the chromosome number by one is far greater than that induced by decreasing it an equal amount.

No morphological effects on the individuals with aneuploid numbers were observed. If the chromosome numbers of the fifty plants are considered, there is a slight indication from the numbers that 29-chromosome plants are more frequent than plants with 27 chromosomes. The counts of somatic chromosome numbers in a larger sample, however (table 2), indicate that no large difference, if any, exists.

TABLE 1

UNIVALENTS IN AUTOTETRAPLOID SECALE CEREALE

CHROMOSOME NO.	CELLS WITH UNIVALENTS								
	0	1	2	3	4	5	6	7	8
	Frequencies								
27	214	123	42	16	3	1	0	0	1
28	2326	959	493	151	53	8	4	3	3
29	253	183	109	39	9	5	1	0	1
Total	2793	1265	644	206	65	14	5	3	5
	Percentages								
27	53.50	30.75	10.50	4.00	0.75	0.25	0.00	0.00	0.25
28	58.15	23.98	12.33	3.77	1.33	.20	.10	.09	.09
29	42.17	30.50	18.17	6.50	1.50	.83	.17	.00	.17
Average	55.86	25.30	12.88	4.12	1.30	0.28	0.10	0.06	0.10

TABLE 2

DISTRIBUTION OF SOMATIC CHROMOSOME NUMBERS

Somatic chromosome no.	26	27	28	29	30	31
Frequency	0	9	82	9	1	0

It is of interest to consider meiosis in the individual with 30 chromosomes. If chromosome additions are deleterious at meiosis, this individual should have a more aberrant meiosis than the 29-chromosome plants. Meiosis in this plant was extremely irregular, asynapsis was most frequent, and fragmentation occurred in parts or all of most cells to a degree which made measuring the asynapsis impossible. Unfortunately no 26-chromosome plant was found, so the comparisons could not be extended to include deficiency as well as duplication for two whole chromosomes, with respect to the tetraploid euploid number. The observations on this 30-chromosome plant are merely suggestive, since the plant could have had some special constitution not frequent among the many other possible 30-chromosome combinations.

The hypothesis that the asynapsis is the result of homologous attractions so balanced among three chromosomes that the center one moves toward neither homologue but remains "suspended" between them can be investigated somewhat in these data. If such were frequently the case, then the asynapsis owing to all other reasons would have

superposed on it asynapsis for two and four chromosomes; that is, the classes with even numbers of chromosomes would be weighted to some degree by this "indecisive" homology. The actual data show no irregularities in the frequency distributions of numbers of cells and numbers of univalents (fig. 9). The absence of any tendency toward higher frequencies in the even-numbered classes seems clear from this graph. The regularity of the frequency distribution would seem to indicate that the asynapsis is a phenomenon which has a random basis and that this is related to the regression coefficient—0.40878 for the 28-chromosome plants. If the three groups are plotted separately, the regularity of the distributions is wholly consistent. The lines or curves have different slopes, but are equally without interruption in approaching the X axis.

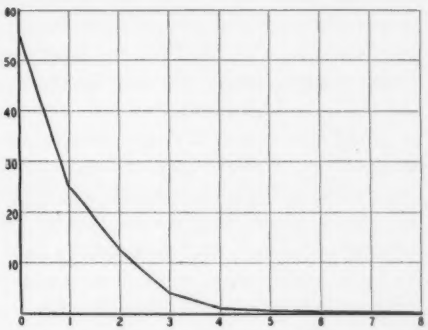


FIG. 9.—Percentages of cells (based on 5000) plotted against number of laggard univalents at anaphase I.

CHROMOSOME BRIDGES

The tetraploid microsporocyte differs from its ancestral diploid further in that its chromosomes possess structural alterations which become obvious at the first meiotic anaphases. An adequate examination, which included several thousand cells, indicated that the diploid had no such chromosome differences. The differences are those in which segments of the threads become rearranged or reduplicated so that bridges extend between bivalent halves at anaphase I. There are two simple ways in which these changes may be imagined to have occurred. The stresses within the tetraploid microsporocyte may be greater in some instances than the threads can withstand, and thus the threads may be ruptured and then reunite in a different order. The unusual homologous attraction among four chromosomes may sometimes compel non-homologous pairing, which is followed by crossing-over or some other break-producing mechanism. The reunion from the crossing-over or breaking could produce a different arrangement of chromosome segments and consequently bridges at meiosis in the next generation.

These bridges are numerous and of various types. The two most frequent may be seen in figures 2 and 8. The uppermost bridge in figure 2 is a simple one which extends from insertion point to insertion point. It has no chromosome fragment near it. The bridge in figure 8 is similar, except that it has a small fragment beside it. This bridge and fragment are simply explained by assuming an inversion within the chromosome arm of one homologue. The size of the inversion is indicated by the size of the acentric fragment. The inversion which produced the upper bridge in figure 2 is similarly explicable, except that it probably was produced by a three-strand double cross-over. Such a cross-over would leave no acentric fragment. It would require a much larger inversion than that in figure 8. The lower bridge in figure 2 is directly comparable with that in figure 8. Superficially this bridge seems to extend between three chromosome arms at one

pole and four at the other, an anomalous arrangement. But what appears to be a chromosome arm at the left of this bridge is probably a fragment, comparable in size with that of the inverted segment. Such fragments frequently pass toward one pole and are thus included in the telophase nucleus. It is clear that this inversion must have involved nearly a whole chromosome arm. These bridges can also be accounted for by assuming duplications instead of simple inversions, as has been done here.

A different kind of bridge may be seen in figure 4. This is stretched between two univalent halves. It can be explained simply by postulating a stickiness at the chromosome ends, such as is known to be present when ordinary bivalent bridges occur without apparent cause. It can also be accounted for on the basis of a rupture in the thread during diakinesis, when the chromosome may have been associated. It is not difficult to imagine that the forces which repel the pairs of threads may infrequently produce complete disjunction and rupture the threads in the unusual autotetraploid microsporocyte. According to McCLINTOCK (1), such a rupture would presumably fuse at the point of the break and thus produce a dicentric univalent chromosome. This would form a bridge at anaphase I and would break at telophase, when fusion would again occur. In maize (1) this breakage-fusion-bridge cycle persists through the gametophytic and endosperm divisions. The hypothesis that the original break occurs by chance at diakinesis is supported by the fact that these bridges were present in all plants in approximately the same very low frequency. If this bridge parallels the similar ones in maize, it would appear last in the division preceding zygosis, after which the chromosome would appear normal.

In figure 7 may be seen a bridge at second anaphase. This one could be produced in the breakage-fusion-bridge cycle started during the first anaphase by inversion bridges, or bridges similar to the univalent bridge just described.

CHROMOSOME LOSS

One of the more unusual phenomena in this autotetraploid is that of chromosome loss. By this is meant the absence of whole chromosomes from the microsporocyte at anaphase I. The loss may include one to many whole chromosomes. A cell with such loss may be seen in figure 6. This cell has 8 chromosomes at one pole, 7 at the other, and a univalent dividing on the plate. The example was taken from a plant with 28 somatic chromosomes.

The data on this kind of cell indicated that practically all the plants must have produced such cells. Of the fifty plants examined, thirty-three had cells deficient for one or more chromosomes. In most instances these were losses for more than 5 chromosomes. It seems probable that among the many hundreds of anthers not examined were patches of deficient cells. The data were secured from two or three anthers from each plant and consequently do not constitute a reliable measure of the actual frequency of such deficiency.

The samples did indicate that in many instances the losses occurred several cell generations before meiosis. The patches of deficient cells included approximately thirty cells in some instances, indicating that the original loss occurred five cell generations before meiosis. In other instances the deficient cells were in pairs, an indication that the loss occurred in the mitosis immediately preceding meiosis. No exact and critical data were secured on the earliest generation at which the loss occurred, because the loss itself

could have resulted in a chromosome unbalance with detrimental effects on mitosis. Furthermore, if this were not true, the technique used in preparing these slides (crushing the anther and its contents) would not be expected to keep the deficient patches intact, and thus a more recent origin than the actual one would be indicated by the cell count. It is also possible that the loss could be progressive—the original loss being followed by further ones. Some evidence for this was found in the preparations. In some islands of deficient tissue were found smaller islands of cells with more extreme deficiency. This is most easily explained by assuming a progressive loss. There was little uniformity in the magnitude of the losses. Table 3 presents data from various cells selected at random.

The losses did not seem to be extremely deleterious to mitosis, since the islands frequently included many cells. In general, however, the more extreme losses were present in the smaller islands. The most extreme losses would be expected to leave the cells with full haploid complements. Even this should be a very severe loss in a plant like rye, which is extremely heterozygous. The actual extreme cases, however, represent losses which leave the cells with less than the haploid complement. Many anaphase cells seemed to possess this excessive reduction, but they have not been tabulated, since the metaphases alone can be safely used in this connection. In all instances except one these were found as single cells. The metaphase configurations are presented in table 3. The single exceptional instance may be seen in figure 5. This shows two adjacent cells, each of which has an identical complement of one trivalent and three univalents, indicating that the original cell, one cell generation removed, was deficient for three whole chromosomes (about 43 per cent of its basic chromosome complement or 79 per cent of its original complement) and still managed to go through a complete mitosis. The two cells illustrated could be seen to have their chromosomes oriented for another division, as is clear from the trivalents, before they were flattened for photographic purposes.

TABLE 3

METAPHASE CONSTITUTIONS OF DEFICIENT CELLS 2n=28

Univalents	Bivalents	Trivalents	Deficiency	Percentage deficient
12	6	2	8	29
3	6	0	19	68
4*	2	0	22	79
5	5	0	18	64
12	2	0	14	50
5	2	0	21	75
2*	2	1	23	82
4	4	1	19	68
4	3	0	21	75
1	7	0	20	71
0*	6	0	22	79
1	5	1	21	75
3*	0	1	24	86
5	2	5	16	57
1 and 2 quadrivalents*	.....	.....	25	89

* Indicates hypoploid cells.

These cells with viable hypoploid complements resemble those found in *Tradescantia* (4), in which microspores deficient for three whole chromosomes developed to the metaphase stage of the first microspore division. This case in *Tradescantia*, and comparable ones in other material, were explained by assuming that gene products from normal contiguous cells diffused into the deficient cells and afforded enough substance to permit normal development. This seems to be the simplest explanation of viability with extreme deficiency, and it probably accounts for the tolerance of deficiency in female gametes, which are con-

tiguous to normal tissue, while male gametes are isolated by heavy walls. The two cells deficient for three chromosomes constitute an instance in which the diffused material was sufficient to make the cell normal enough to divide. The possibilities should be considered that all chromosomes are not equally necessary, however, and that the lack of certain ones may be partially compensated for by duplication of others. The three missing chromosomes may be constituted of much nonessential or inactive material, and the trivalent may replace much of what they contribute to the cell. It would therefore be unsafe to state that a full three-sevenths of the genes and their products are missing. That a great deficiency does exist, and that it is compensated for by the diffusion of gene products from adjacent and contiguous cells, seems reasonable.

ANAPHASE DISTRIBUTIONS

The chromosome behavior just described was such as would produce gametes with anomalous chromosome numbers. Another source of such irregular numbers is the division or disjunction mechanism itself. Any tendency which the spindle may have toward distributing metaphase chromosomes unequally at anaphase would produce microspores with different numbers. The mechanism is extremely precise in the diploid. It may be equally precise in the tetraploid, or it may be affected by chromosome unbalance in the tetraploid, or it may simply be altered in its precision by tetraploidy itself. The data necessary for considering this problem can be secured with reasonable exactness and adequacy. Their dependability is impaired somewhat by the facts that no estimate of the frequency of nonlagging univalents could be secured and that a trivalent can divide as equally as possible and still produce an unequal anaphase. The first source of error must be accepted; the second will compensate itself in many or most cells. The cells in which two or more trivalents disjoin so that one pole receives twice as many chromosomes as the other are those in which the anaphase counts will imply a degree of irregularity in the spindle which does not exist. From these considerations it would seem safe to state that the degree of spindle irregularity measured is the minimum irregularity, or an underestimated regularity.

TABLE 4

ANAPHASE DISTRIBUTION IN AUTOTETRAPLOID SECALE CEREALE

PLANT CHROMOSOME NO.	DEGREE OF IRREGULARITY				
	0	1	2	3	4
	Frequencies				
27	313	82	5	.....	.....
28	3138	821	38	2	1
29	491	101	7	1	.....
Total	3942	1004	50	3	1
	Percentages				
27	78.25	20.50	1.25	.....	.....
28	74.85	20.52	0.95	0.05	0.03
29	81.83	16.83	1.16	1.00	.....
Average	78.85	20.08	1.00	0.06	0.02

In summarizing the data (table 4), the following procedure has been used. If the anaphase chromosome numbers are equal in a 28-chromosome plant, or differ by one in a 27- or 29-chromosome plant, the division has been classified as 0 degrees irregular. A difference of one in the former class, or two in the latter classes, has been classified as 1 degree irregular. In

this manner the divisions have been classified up to 4 degrees irregular. Where univalents occurred, their number was subtracted from the total number and the irregularity then determined as before—by the degree it departed from the best possible disjunction. An inspection of the percentages (table 4) seems to indicate that the spindle mechanism must be extremely precise—perhaps as exact as it is in the diploid. The average percentage of cells with 1 degree of irregularity is 20 per cent. This whole class can be accounted for merely by assuming that in one cell in five a trivalent and its corresponding nonlagging univalent were unequally distributed and uncompensated by similar behavior in the opposite direction by another trivalent. This means that such behavior need occur but once in 140 chromosome associations, if no other source of irregularity exists. However, another source of irregularity does occur. Two nonlaggards at the same pole would produce an unequal result. It would seem that the spindle mechanism is perhaps as precise as that in the diploid, and that the irregular anaphases are largely the results of chromosome associations which can produce irregular results from regular disjunctions, that is, trivalents and nonlagging univalents. Since there are but 20 per cent irregular cells, and since this can be accounted for by assuming a very low percentage, 0.71 per cent, for random phenomena which are known to occur, it would seem more probable that the spindle mechanism is precise and that 0.71 per cent indicates the combined frequencies of nonlaggards at the same pole, trivalents disjoining in the same direction, and trivalents and nonlagging univalents producing 3/1 distributions.

The data in table 4 seem to indicate further that the group with 29 chromosomes had more regular anaphase distributions than had the other two groups. The X^2 for this class compared with the 28-chromosome class is *ca.* 3.8, or just below the 5 per cent value.

Another separation of the data, in which the number of univalents per cell is tabulated against the degrees of irregularity, is presented in table 5. The table indicates that the distributions are not correlated with univalent number.

TABLE 5

ANAPHASE DISTRIBUTIONS IN AUTOTETRAPLOID SECALE CEREALE

No. of univalents in cell	Degree of irregularity				
	0	1	2	3	4
	Frequencies				
0	2171	596	25	1	.....
1	1064	188	11	0	2
2	469	165	9	1	.....
3	172	32	2	.....	.....
	Percentages				
0	77.73	21.34	0.89	0.04	.....
1	84.11	14.86	0.87	0.00	0.15
2	72.83	25.62	1.40	0.15	.....
3	83.50	15.53	0.97	.....	.....

Sources of micronuclei

Recently the relation between anaphase laggards and tetrad micronuclei has received much attention (2). According to the scheme presented by other writers, each laggard splits at anaphase I, each half passing to its nearest pole. At anaphase II these halves are distributed at random to the poles. If these events occurred with regularity, deficient and duplicated quartet members would be formed but no micronuclei. But the half

univalents from the laggards at division I do not all reach the poles at division II soon enough to be included in the telophase nucleus, and thus micronuclei are formed. If the frequency of laggards is known, certain predictions about the distributions and frequencies of micronuclei can be made. Thus, one laggard should produce as a maximum two micronuclei, each of which must occur in a different microspore. Two laggards would produce four micronuclei, with two as the maximum in a microspore. The data secured in these observations indicate that no such regularity occurs, since micronuclei are found in unexpected frequencies and combinations. That many micronuclei are wholly unrelated to simple anaphase laggards may be seen in several of the photomicrographs included here. Figure 2 shows a division with one laggard which has split and is passing to the poles, while two bivalents are so delayed that the telophase nuclei will probably be formed before the bridges break and allow the bivalent halves to be wholly included. Figure 8 shows three laggards splitting, while a bivalent has an inversion or duplication bridge, the fragment of which will form a micronucleus. Figure 4 is of a univalent which has one set of ends joined, presumably from a previous rupturing of the ends, so that it may well split into three chromatin masses instead of two. Such breaks are not infrequent, wherein the two main masses of chromatin, the univalents, are toward the poles while the bridge remains in the center near the cell plate, and in some instances is divided in two by the plate. The data also indicate that, while in general the frequency of laggards observed at anaphase is distributed more or less in a regular fashion (that is, a distinct mode exists), it is also true that at both metaphase and anaphase exceptional cells occur which have an unexpected number of univalents and laggards. Such cells would produce tetrads with many more than the predicted numbers of micronuclei. Another source of anomalous tetrads may be metaphase cells with a reduced number of chromosomes. Such deficiency extends from losses of single ones to a loss of 22 chromosomes, or 79 per cent of the individual's initial complement. Although these cells usually divide normally at division I, they have a higher percentage of univalents than normal microsporocytes. Furthermore, while they do complete the first division normally, many of the nuclei which result from this division enter the second division with fewer than a normal haploid complement of chromosomes and may be presumed, in some instances at least, to yield an abnormal contribution to the tetrad members. There are therefore many ways in which micronuclei may be formed which are wholly or partially independent of the laggards which occur at anaphase I. This conclusion is supported by observations on the sizes of micronuclei, which range continuously from extremely small chromatin dots which are hardly visible to large reticulate masses which must include several chromosomes. In several instances single quartet members had two isodiametric nuclei. It seems clear that single anaphase laggards cannot vary in size from representative micronuclei down to the smallest ones, which have a volume less than one-tenth of average. It seems probable that any scheme for predicting the numbers and dispositions of tetrad micronuclei from the numbers of anaphase laggards can predict only the minimum numbers and simplest dispositions. In some instances, as in the present material, these predictions would have only a very approximate, if any, reliability.

Summary

1. Meiosis in autotetraploid *Secale cereale* is characterized by multivalent associations, inversion or duplication bridges, univalent bridges, univalent laggards and nonlaggards, irregular anaphase distributions, and chromosome loss.

2. The degree of anaphase irregularity is so low as to indicate that the spindle mechanism is as precise in the autotetraploid as in the diploid.

3. Unequal anaphases are produced by nonlagging univalents and coincidences in the divisions of trivalents.

4. Chromosome loss seems to interfere little with mitosis until the reduction leaves little more than the haploid number. Hypoploid cells were capable of division, the extreme instance being the division of a cell deficient for three whole chromosomes. The loss occurs frequently, usually within five or six cell generations before the first meiotic division, and may be followed by additional loss within the islands of deficient cells.

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COMPOSITION OF THE TOMATO PLANT AS INFLUENCED BY NUTRIENT SUPPLY, IN RELATION TO FRUITING[1]

D. I. ARNON AND D. R. HOAGLAND

Introduction

This paper presents the results of some biochemical studies carried out in the last several years as part of an investigation of the relation of the nutrient medium to the growth and fruiting of the tomato plant. Incidental to large-scale experiments involving the use of artificial culture media and soils (3), an opportunity presented itself to explore the effects of different nutrient environments on the composition of fruit and foliage of plants maintained under the same climatic conditions. In addition, an attempt was made to relate the effects of fruit-bearing to the organic and inorganic composition of plants grown in complete as well as in potassium- and phosphorus-deficient nutrient solutions.

METHODS.—The plants were grown by the large-scale culture methods already described in detail (3). Unless otherwise noted, black iron tanks with a capacity of 460 liters were used in all nutrient-solution experiments. The soil and sand cultures referred to are those previously described (3). The methods of germinating seeds and the nutritional history of seedlings prior to transplanting to the experimental medium are—except for the stated differences—also the same as those given in the earlier publication.

The seven nutrient solutions used are presented in table 1. Iron was added once or—as the plants became larger—twice weekly at the rate of 0.66 cc. of 0.5 per cent iron tartrate or iron sulphate solution to a liter of nutrient solution.

TABLE 1

COMPOSITION OF NUTRIENT SOLUTIONS IN TERMS OF MOLAR CONCENTRATIONS OF COMPONENT SALTS*

Salt	Nutrient solutions						
	I	II	III	IV	V	VI	VII
KNO_3	0.006	0.010	0.001	0.010	.....	.....	.....
K_2SO_4	.....	.....	.....	.....	0.005	.....	0.005
$NH_4H_2PO_4$	0.001	0.002	0.002	0.0002	0.002	0.002	.....
$(NH_4)_2SO_4$	.....	.....	.....	0.0009	.....	.....	0.001
$Ca(NO_3)_2$	0.004	0.003	0.0075	0.003	0.006	0.006	0.006
$MgSO_4$	0.002	0.002	0.002	0.002	0.002	0.002	0.002

* All solutions except nos. I and II received the supplementary micro-nutrient solution A_5 (2); solutions I and II received the supplements A_4 and B_7 (1), except that in the experiment represented in table 4 solution II received only the supplement A_5.

The standard procedures previously referred to (3) were used in analyzing the nutrient solutions and in determining the inorganic constituents of plants. Total nitrogen was determined by the Kjeldahl method.

For the analysis of organic constituents, the plants were boiled, immediately after harvesting, for 15–20 minutes in a suitable volume of 95 per cent ethyl alcohol, computed to give an effective concentration of 80 per cent alcohol after dilution by the plants. Extraction was completed in a Soxhlet apparatus. The extract was analyzed for total soluble ni-

[1] C. M. JOHNSON and W. E. FRATZKE have rendered valuable analytical assistance in the course of this study.

trogen by the reduced-iron modification of the Kjeldahl method; it was also analyzed for total and reducing sugars (5). The residue from the Soxhlet extraction was analyzed for starch (6) and for total nitrogen by the Kjeldahl method. The total nitrogen of the residue multiplied by the factor 6.25 is reported as protein nitrogen.

All analyses except those of the alcoholic extract were carried out on dried and ground material. Unless otherwise specified, each fruit sample was prepared for analysis by selecting from each treatment enough uniform fruits of the same degree of ripeness to give 1 kg. of fresh weight. Leaf samples were likewise prepared by pooling the detached leaves from each treatment and taking for analysis after thorough mixing a sample of about 100–200 gm. of fresh weight. The plant material was dried at 70° C. in a ventilated oven and was ground in a Wiley mill to pass a 60-mesh screen.

Results

Effect of nutrient media on inorganic[2] composition of fruit

Table 2 presents the results of analyses of tomato fruits harvested from plants grown side by side in the same greenhouse in soil, water, and sand culture. As shown by the previously reported (3) large yields from these cultures, all three media had an adequate supplying power for nutrients, and the plants were grown under climatic conditions conducive to fruitfulness. The data in table 2 indicate that in these circumstances the inorganic composition of fruit grown in the three different media was relatively constant, with the exception of magnesium. No marked variation was found in the water content of the three kinds of fruit grown in the three types of media.

[2] Inorganic composition and similar terms are here used with reference to elements introduced into the plant through absorption by roots, whether or not these elements subsequently enter into organic combinations.

Effect of total concentration and relative composition of nutrient solution on composition of fruit and foliage

The relation of the composition of the nutrient medium to that of the plant was further explored, with the aid of the nutrient solution method, which easily lends itself to controlled variation of the external supply of nutrients. The influence of total concentration of the nutrient medium was followed in an experiment, the results of which are presented in table 3. The plants, after having been grown for several weeks in a basic nutrient solution (solution II, table 1), were divided at the onset of the first flower clusters into three groups of twenty plants each and transferred into three nutrient solutions. The first group of plants continued to grow and set fruit in a basic nutrient solution of the same composition as the original one; the second group was placed in a solution of twice, and the third group in a solution

TABLE 2

Effect of nutrient media on composition of greenhouse tomatoes, variety Lloyd Forcing (p.p.m. of fresh weight)

	Water culture*	Sand culture*	Soil
K	2238	2175	2085
P	321	352	239
Ca	99	94	104
Mg	69	54	86
N	1270	1230	1090
Moisture (%)	95.3	95.9	95.1

* Solution I (table 1) used as source of nutrients.

of three times, the concentration of the basic nutrient solution.

Analysis of the ripe fruit from the three treatments (table 3) showed that, despite the differences in the total concentration of the external nutrient medium, the inorganic composition of the fruit remained essentially unaffected. There was no distinct relation between the water content of the fruit and the total concentration of salts in the external medium.

TABLE 3

EFFECT OF CONCENTRATION OF NUTRIENT SOLUTION ON COMPOSITION OF GREENHOUSE TOMATOES, VARIETY SUTTON'S BEST OF ALL (P.P.M. OF FRESH WEIGHT)

	Basic*	Twice basic concentration	Thrice basic concentration
K	2950	3040	2780
P	380	369	379
Ca	34	49	42
Mg	105	106	99
N	1652	1515	1690
Moisture (%)	95.7	94.6	94.9

* Nutrient solution II (table 1).

This relative constancy of the composition of the fruit is also apparent from the data given in table 4. In this experiment the plants were grown in three solutions of approximately the same total concentration but differing in their supply of potassium and phosphate ions. In the control solution the potassium concentration was allowed to fluctuate between 400 and 200 p.p.m. and that of phosphate between 200 and 100 p.p.m. In the low-potassium solution the potassium concentration was maintained between 40 and 0 p.p.m.; the solution was not allowed to remain without potassium for any appreciable length of time, and on approaching the zero point it was restored to a concentration of 40 p.p.m. In the low-phosphate treatment the phosphate concentration was maintained between 20 and 0 p.p.m. but was never allowed to remain at zero. Thus the low-potassium and low-phosphate treatments are to be regarded, not as deficient cultures but as nutrient media which have been supplying potassium and phosphate, respectively, from a low concentration. All three solutions were analyzed at frequent intervals, and salts were added as required.

TABLE 4

EFFECT OF LOW K AND PO_4 ON MINERAL AND MOISTURE CONTENT OF TOMATO FRUIT AND FOLIAGE, VARIETY SUTTON'S BEST OF ALL (P.P.M. OF FRESH WEIGHT)

	Fruit			Leaves		
	Control*	Low K	Low PO_4	Control*	Low K	Low PO_4
K	2650	2230	2490	5120	3900	5300
P	333	333	350	1318	1321	646
Ca	42	51	39	2770	3500	2320
Mg	105	88	94	1060	730	800
N	1530	1460	1480	5320	5610	5240
Moisture (%)	94.9	95.3	95.0	87.9	87.8	88.3

* Control treatment received nutrient solution II; low K and low P received solutions III and IV, respectively (table 1).

The effect of the different levels of external supply of potassium and phosphate on the composition of the fruit was contrasted with that on the composition of the foliage. Mature but not senescent leaves were picked, generally from about 1–3 feet from the tip of the plant. The plants were trained to a single stem and allowed to extend vertically up to a height of 10–12 feet. No distinction was made in this analysis between leaf blades and petioles.

As shown in table 4, the low phosphate supply in the nutrient medium was associated with marked reduction (approximately half) of the phosphorus content of the leaves, while that of the fruit remained about the same as in the control.

Despite the known interrelations between nitrate and phosphate absorption, the nitrogen content of the low-phosphate leaves remained essentially unchanged. This can probably be attributed to the very liberal supply of nitrogen in all the cultures. As to the potassium content of the plant, both leaves and fruit showed a reduction in the low-potassium treatment as compared with the control, but the reduction was more marked in the leaves than in the fruit. The fruit from the low-potassium treatment was characterized by softening on the vine before attaining full color. It is possible that the small decrease in the potassium content as a result of a low external supply is associated directly or indirectly with firmness of the maturing fruit. On the other hand, the larger differential of potassium content in the leaves may have been related to modified production of organic metabolites in the leaf, which influenced the character of the fruit.

The data in table 4 invite comparison as to the relative proportions of the inorganic constituents of leaves and fruit. The outstanding difference lies in the markedly lower concentration in the fruit of calcium and magnesium in relation to potassium. These and subsequent data (table 5) indicate that this relative proportion of bases is on the whole a fixed characteristic of the fruit, and it does not appear to be readily influenced by fluctuations, short of total deficiencies, in the external supply of nutrients.

The mineral composition of the fruit was modified in essential respects only when the plants were grown, for the period subsequent to flowering, in a nutrient medium totally deficient in some nutrient element. Table 5 compares the analyses of fruits, both young (green) and mature, grown in nutrient solutions deficient either in potassium or in phosphate, with fruits grown simultaneously in a complete solution. The number of fruits from each treatment included in the analysis varied from nine to eighteen for the ripe and from sixteen to fifty-nine for the green fruit. Each analytical value in table 5 represents the average of two independent but closely agreeing determinations made on samples secured from parallel cultures. The conditions under which the plants were grown will be discussed in the following section, and it will suffice to state at this point that the plants, later identified as fruitful in the defloration experiment, were kept in the respective media from the onset of the first flower cluster. Corresponding data for the composition of the foliage will be found in figures 4, 5, and 7.

TABLE 5

EFFECT OF POTASSIUM AND PHOSPHORUS DEFICIENCY ON MINERAL AND MOISTURE CONTENT OF TOMATO FRUIT, VARIETY MARGLOBE (P.P.M. OF FRESH WEIGHT)

	Ripe			Green		
	Complete	Minus K	Minus PO_4	Control	Minus K	Minus PO_4
K	2645	1150	2255	2395	1420	2210
P	529	570	116	473	707	112
Ca	42	60	46	34	130	51
Mg	106	94	130	118	123	147
N	1762	2660	1510	1417	2930	1385
Moisture (%)	94.3	93.8	95.5	94.4	93.4	96.5

Of course, the "ripe" fruit produced by the potassium- and phosphorus-deficient plants was in no sense of marketable quality. The fruit was of small size, poorly developed, and in the minus-phosphate cultures it consistently exhibited symptoms closely reminiscent of blossom-end rot. As the subsequent discussion will show, the potassium and phosphorus of the fruit in the deficient cultures was derived from the reserves of these elements in the foliage, absorbed

during the preliminary period when all plants were grown in a complete nutrient medium. The great differences in the mineral content of the fruit shown in table 5 cannot therefore be taken as indicative of the normal range of variation, but rather of redistribution of the potassium and phosphorus reserves within a plant in which the development of the fruit has been initiated before the external supply of nutrients has become deficient. The previously stated conclusion about the relative constancy of the mineral composition of normal fruit remains unaffected by the present data.

Table 5 shows that the fruit from the minus-phosphate cultures had more than a fourfold decrease in its phosphorus content as compared with the fruit from the complete nutrient solution. Likewise, the potassium content of the tomatoes from the minus-potassium treatment was less than half that in the normal fruit. These differences in composition were apparent not only in the ripe (fully-colored) but also in the immature green fruit. (This included assorted sizes, however, and cannot be regarded as physiologically uniform.) A high calcium content was found in the potassium-deficient fruit, particularly in the young fruit. It seems reasonable to expect, from the known interrelations of cations, increased calcium migration to the potassium-deficient fruit, especially to the young developing fruit, which has a high capacity for solute absorption. The potassium-deficient fruit showed, particularly when the results were expressed on a fresh-weight basis, an unusually high nitrogen content.

The subsequent increase in the size of the green fruit, accomplished largely by the enlargement of the size of the existing cells and accompanied by the intake of water, would explain the decrease of calcium on a concentration basis in the ripe fruit. The higher calcium content in the potassium-deficient plants was not confined to the fruit but was also evident in the foliage (figs. 4, 5). This points to an increased calcium absorption (per unit weight of plant) from the nutrient substrate by the plants grown in the potassium-deficient solutions. The phosphorus-deficient had a higher magnesium content than the normal fruit.

Defloration experiments

The contrast between the composition of fruit and foliage has suggested the desirability of investigating the effect of fruit-bearing on the inorganic nutrient distribution in the plant. This was studied under three conditions of external nutrient supply: complete, minus-potassium, and minus-phosphate nutrient solutions (V, VI, and VII, respectively, table 1). With the exception of the deficient nutrient, the concentration of all the other elements, save sulphur, was the same in the three solutions. The sulphate ion was used in balancing the ionic charges.

Tomato plants of the Sutton's Best of All and Marglobe varieties were germinated in sand and grown in a complete nutrient solution (half strength of solution II, table 1) for a period of about 5 weeks, until the first flower clusters emerged. The plants were then selected for uniformity and divided into three groups of twenty-four plants each. Each group of twenty-four constituted one nutrient treatment, which comprised four metal tanks of solution, each tank having a capacity of 115 liters and supporting six plants. Within each treatment in turn, half the plants, that is, those in two tanks, had their flowers removed as soon as they emerged. The plants in the remaining two tanks were allowed to set

fruit.[3] The flowers on the fruitful plants were tapped regularly to insure pollination.

EFFECTS IN COMPLETE NUTRIENT SOLUTION.—As shown in figure 1, the fruitful plants in the complete nutrient made excellent growth and produced a relatively heavy crop of fruit. Unusual growth effects, however, were observed in the deflowered series. After several weeks the stems, petioles, and leaves became much thickened, rootlike structures appeared on the stems, and new growing points emerged prolifically from the leaf axils (fig. 2). These growing points were soon removed, together with all side branches, thus confining growth to the main stem. The characteristic thickening of the stem tapered down to the terminal growing point, which withered and died (fig. 3). These symptoms were reproduced in a second experiment carried out in a different season.

The profound morphological changes as a result of defloration were associated with a change in the organic composition of the plant (table 6). Samples of leaves were taken from the deflowered and fruitful plants at the conclusion of the experiment, 93 days after defloration. They consisted of the second and third leaves below the tip of each plant. The leaf blades and the petioles (which included the midribs) were analyzed separately. In addition, analyses of stems were made, for which the terminal 2-feet sections were used. Samples from plants grown in one tank were analyzed collectively, and since each deflowered and fruitful series comprised two tanks, this provided a replication of the analyses, which gave close agreement.

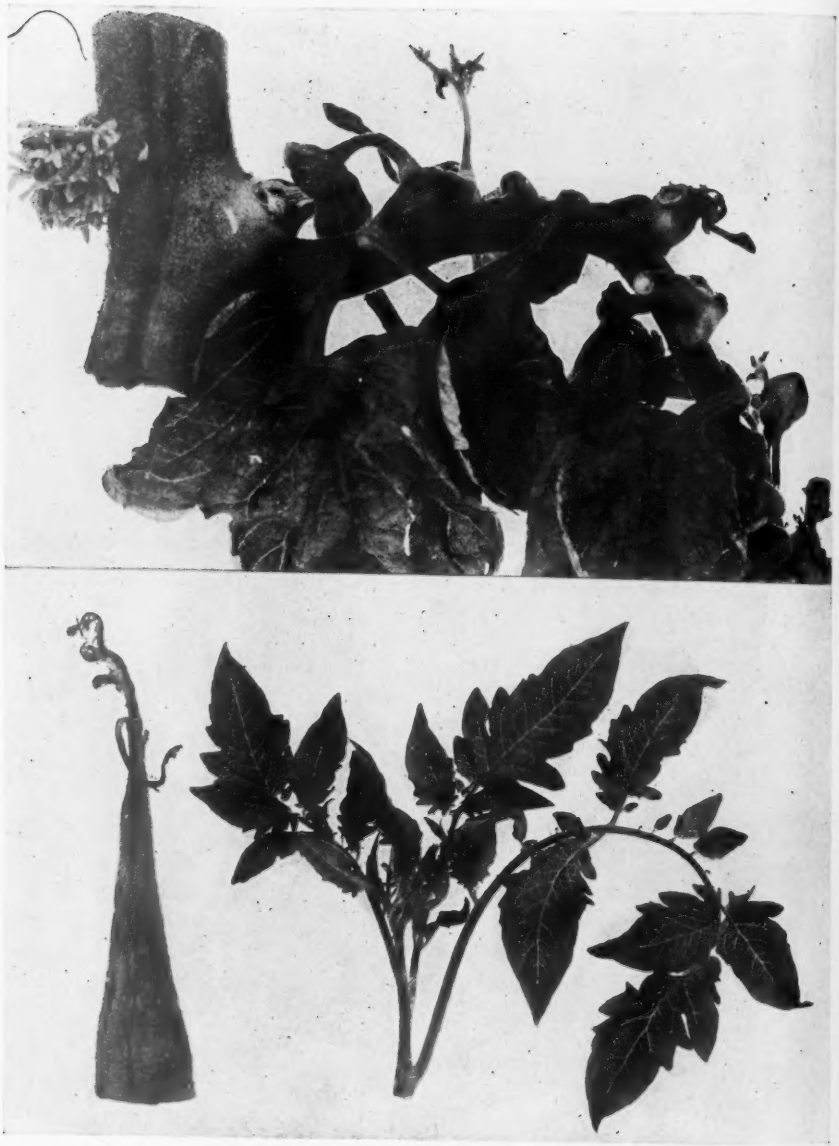
As shown in table 6, the differences in composition between the deflowered and fruitful plants were particularly notable in the stems and petioles. Common to all tissues from the deflowered plants was the marked accumulation of starch, especially pronounced in the stems,[4] in which

FIG. 1.—Fruitful plants in complete nutrient solution.

a sevenfold increase over the fruitful plants was found. It is to be noted that starch was accumulated under climatic conditions favoring growth and in the absence of any nutrient deficiency. The petioles and the stems of the deflowered plants also had a higher concentration of total sugars and of total soluble as well

[3] The former will be referred to as deflowered and the latter as fruitful.

[4] Confirmed in histological examination of the tissue by Dr. CHARLES HEIMSCH.



FIGS. 2, 3.—Fig. 2 (above), section of stem and leaf from deflowered plant grown in complete nutrient solution; note thickening and emergence of rootlike structures from stem and new growth at leaf axils. Fig. 3 (below), terminal growing point in deflowered (left) and fruitful (right) plant, grown in complete solution.

as protein nitrogen. Only negligible amounts of nitrate nitrogen were found in the alcoholic extracts of either fruitful or deflowered plants. In view of its continuous supply in the nutrient medium, the absence of nitrate in the tissues points to its prompt utilization—a course of events to be expected from the favorable supply of carbohydrates. Except for starch, the leaf blades of the deflowered plants showed no marked differences from those of the fruitful plants in the organic constituents for which analyses were carried out. Unlike the stems and petioles, the protein content of the "fruitful" leaf blades was found to be higher than that of the "deflowered" ones. The deflowered plants made more vegetative growth than the fruitful ones (table 7).

TABLE 6

ORGANIC CONSTITUENTS OF DEFLOWERED (D) AND FRUITFUL (F) TOMATO PLANTS GROWN IN COMPLETE NUTRIENT SOLUTIONS (PERCENTAGE FRESH WEIGHT)

	Leaf Blades		Petioles		Stems	
	D	F	D	F	D	F
Starch	1.91	1.35	0.35	0.13	1.05	0.15
Total sugars	2.19	2.05	2.67	1.81	3.31	2.95
Reducing sugars	2.01	1.92	2.08	1.72	2.51	2.65
Soluble N*	0.056	0.047	0.082	0.034	0.148	0.037
Protein N	1.93	2.51	0.89	0.71	1.05	0.69

*Only negligible amounts of nitrate nitrogen present.

Earlier in the growing period, 46 days after defloration, samples of leaves were taken for the determination of potassium, calcium, magnesium, and phosphorus. The deflowered plants exhibited at that time the already-described characteristic morphological symptoms but in less extreme form; the withering and dying of the terminal growing points was only at its inception. The leaves were collected in descending order from the tip of each plant and designated by ascendingly consecutive numbers. Leaf number 1 represented the first and number 6 represented the sixth consecutive leaf below the growing point. This method of sampling allowed the tracing of the gradient distribution of potassium, calcium, magnesium, and phosphorus in the plant from the youngest to the mature leaves. Leaves occupying the same position relative to the tip were pooled and analyzed collectively. Separate analyses were made on the leaf blades and petioles, with the latter including the midribs.

TABLE 7

AVERAGE FRESH WEIGHT OF SHOOTS AND ROOTS AT END OF EXPERIMENT (GRAMS)

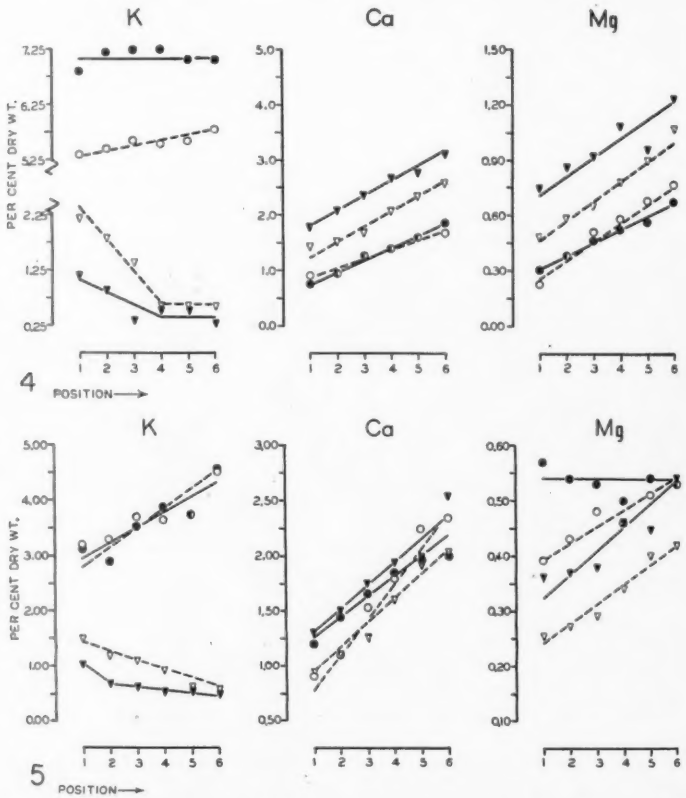
Treatment	Shoots*	Roots
Complete		
Fruitful	1570	339
Deflowered	2160	546
Minus K		
Fruitful	332	171
Deflowered	393	264
Minus PO_4		
Fruitful	302	178
Deflowered	530	259

* Weight of shoots is exclusive of fruits.

The results of potassium, calcium, and magnesium analyses are shown in figures 4 and 5. No outstanding differences are noted between the compositions of the fruitful and deflowered plants, except that the petioles from the latter had a lower potassium content than those from the former. This may possibly be an indication of active transport of potassium to the fruit, which—as shown previously—has a high requirement for this nutrient element. Close similarity was also found in the phosphate contents of the fruitful and deflowered plants (fig. 7).

EFFECTS IN MINUS-POTASSIUM NUTRIENT SOLUTION.—The removal of flowers from plants grown in the potassium-defi-

cient nutrient had a distinct effect on the alleviation of the potassium-deficiency symptoms. It is generally known that plants suffering from potassium deficiency exhibit a breakdown of the older leaves accompanied by a more or less sium-deficiency symptoms were evident near the tip, the deflowered plants made more growth (table 7), and leaves of a corresponding position on the vine appeared more healthy (fig. 6), showing less advanced potassium-deficiency symp-



FIGS. 4, 5.—Fig. 4, petioles; fig. 5, leaf blades: potassium, calcium, and magnesium contents from fruitful and deflowered plants. Position numbers denote descending order of leaves from tip. Full triangles: fruitful, minus K. Open triangles: deflowered, minus K. Full circles: fruitful, complete. Open circles: deflowered, complete.

normal formation of the youngest leaves, near the growing point, which withdraw the potassium from the older leaves. As the deficiency advances, the symptoms appear in progressively younger leaves, and ultimately, of course, the entire plant dies. While in the fruitful plants potastoms in a deflowered plant on a leaf from the same position relative to the tip than on the leaf from the fruitful plant. Figure 6 also shows that the unusual growth proliferations noted on the leaves of the deflowered plants grown in complete nutrient (fig. 2) were much less pronounced

in the potassium-deficient deflowered plants.

Leaf samples for inorganic analysis were taken from the potassium-deficient plants, beginning near the growing point and progressing down, as previously described. The difference in the relative severity of potassium-deficiency symptoms between the fruitful and deflowered plants was reflected in the appearance of the collected leaves. In all twelve deflowered plants, the first and the second leaves (progressing from the growing point down) were free from deficiency symptoms, which were observed on leaves of similar position in some of the fruitful plants. The third leaf was free from deficiency symptoms in nine out of twelve deflowered plants but in only one out of twelve fruitful plants. The fourth, fifth, and sixth leaves were damaged in both the fruitful and deflowered plants (except for one healthy fourth leaf in a deflowered plant).

These symptomalogical differences are in accord with the analytical data presented in figures 4 and 5. The potassium content of the youngest leaves is distinctly higher in the deflowered plants, but in lower (older) leaves it approaches the level found in the fruitful plants. A reversal was noted in the case of calcium and magnesium, which were higher in the fruitful plants than in the deflowered ones. The lower potassium content of the fruitful plants is no doubt

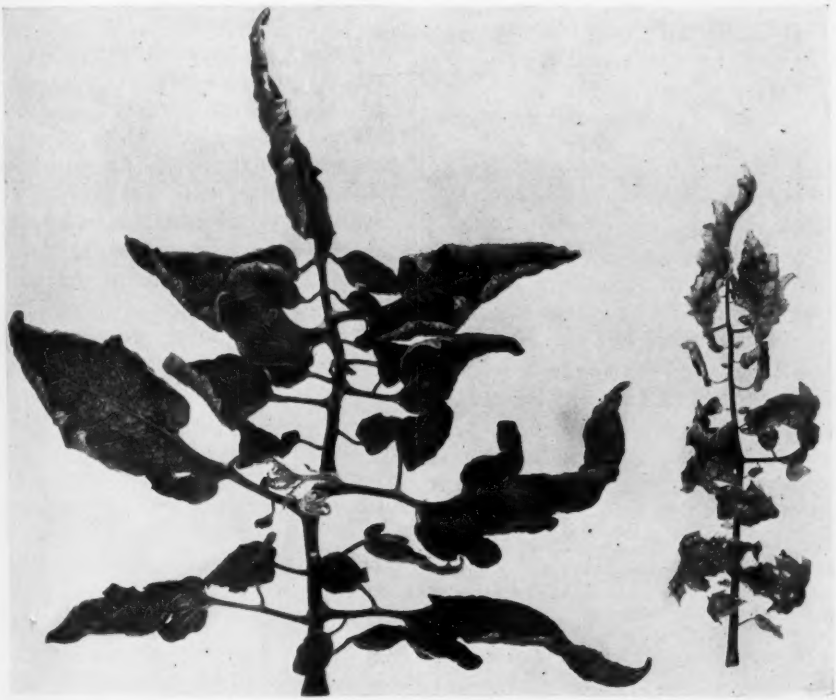


FIG. 6.—Effect of defloration on onset of potassium-deficiency symptoms in plants grown in potassium-deficient medium. Left, leaf from deflowered plant; right, leaf of corresponding position from fruitful plant.

attributable to removal of part of the potassium reserves to the developing fruit. The higher calcium and magnesium contents of the fruitful plants may be due to increased absorption of these cations to compensate for the export of potassium to the fruit. Under these abnormal conditions, calcium and magnesium con-

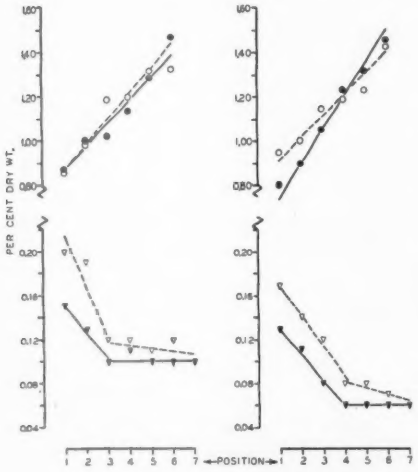


FIG. 7.—Phosphorus content of leaf blades and petioles from fruitful and deflowered plants. Upper and lower left, blades; upper and lower right, petioles. Position numbers denote descending order of leaves from tip. Full triangles: fruitful, minus P. Open triangles: deflowered, minus P. Full circles: fruitful, complete. Open circles: deflowered, complete.

ceivably may have replaced up to a certain point the function of potassium in balancing the organic acids in the leaves. In the absence of knowledge of changes in total calcium and magnesium in the leaves, these views are of course speculative. The data available pertain to percentage values on a dry-weight basis.

Figures 4 and 6 indicate differences in the potassium content of plants grown in complete and potassium-deficient nutrient solution. The former had several times more potassium, both in the fruitful and deflowered plants, than the latter. Moreover, with an abundant external supply of potassium, its concentration in the leaf seemed to increase in the older leaves, whereas the reverse was true in the potassium-deficient plants. As for the calcium and magnesium content, the plants grown in complete nutrient, unlike the potassium-deficient plants, showed no marked difference between the fruitful and deflowered plants.

EFFECTS IN MINUS-PHOSPHATE NUTRIENT SOLUTION.—Defloration of plants grown in a minus-phosphate nutrient solution produced effects on growth and composition similar to those reported for the potassium-deficient plants. The deflowered plants made more vegetative growth (table 7). Phosphorus-deficiency symptoms were more pronounced and more frequently encountered in the fruitful plants for any given leaf position on the vine. The results of phosphorus analyses (fig. 7) show a higher content in the youngest leaves of the deflowered plants than in the fruitful ones. These findings are in harmony with the evidence obtained by means of a radioactive isotope technique, that in the absence of adequate supply of phosphorus for both fruit and foliage the developing fruit draws on the phosphorus present in the leaves (4).

The phosphorus content of the leaves from the control plants, whether deflowered or fruitful, was several times higher than that for the phosphate-deficient plants (fig. 7). Defloration of plants grown in complete nutrient did not markedly affect the phosphorus content of the leaves, at least after several weeks. It will also be recalled that, unlike the deflowered potassium-deficient plants, defloration of the plants grown in complete nutrient did not greatly alter the composition of leaves with respect to inorganic

bases. These facts seem to warrant the conclusion that prevention of fruiting was important in modifying the inorganic content of vegetative tissues only when the external supply of nutrients was limiting. The marked differences found in the composition of leaves from potassium- and phosphate-deficient plants, including the reversal of concentration gradients between the old and young leaves, offer another line of evidence for the physiological validity of establishing potassium and phosphate deficiencies by methods of foliar diagnosis.

Discussion

The analytical data offer ample evidence that the inorganic composition of tomato fruit, as contrasted with the modifiable composition of the foliage, is relatively constant and not easily influenced by fluctuations in the external supply of nutrients, provided that outright deficiencies are absent. On a percentage weight basis the concentration of each of the inorganic nutrients studied is markedly lower in the fruit than in the leaves, but even more striking are the changes in the relative proportions of these constituents in the fruit. Potassium is the dominant element in fruit and—together with nitrogen—one of the two preponderant ones in the leaves. But the ratio of calcium:potassium shifts from approximately 1:2 in the leaves to 1:60 in the fruit and that of magnesium:potassium from 1:5 to 1:25 (computations based on data from the control treatment, table 4). The shifts in the proportions of phosphorus and nitrogen relative to potassium are less drastic but still appreciable. Another contrast with the leaf is the higher magnesium than calcium content of the fruit (on a percentage weight basis). The relative immobility within the plant of calcium as compared with magnesium and other mineral elements will be recalled in this connection (11). Of course, the figures cited apply only to a particular variety of plant grown under a given set of climatic conditions, yet other unpublished data indicate analogous relationships in other tomato varieties grown at various seasons.

A total deficiency, however, of either potassium or phosphorus in the nutrient medium, as contrasted with fluctuations above a minimum supply, is reflected in a decrease of these elements in the fruit, associated with a general growth disturbance and production of abnormal fruit (9). The results of the defloration experiments in the deficient cultures suggest that the developing fruit draws on the phosphorus and potassium reserves of the leaves, which leads to an accelerated breakdown of vegetative tissues. This is consistent with the general view that the regions undergoing most active growth and development, whether vegetative or reproductive, have the highest capacity for absorption and accumulation of inorganic nutrients, including the power of removal from physiologically less active plant organs.

The data, however, are incapable of establishing the mechanism by which the export of nutrients from older leaves in deficient plants to the developing fruit and younger leaves is accomplished. Two general types of mechanisms may be envisaged: one residing in the young tissue, which brings about the withdrawal of potassium or phosphorus from the older tissue through physiological changes in the latter incited by some hormone-like substance; another involving continuous circulation in the phloem of phloem-mobile nutrients (11). The second mechanism would require the further assumption that, in case of deficiency, the youngest tissues have the greatest power of reten-

tion from the phloem system of nutrients, such as potassium and phosphorus.

When a potassium- or phosphorus-deficient plant is deflowered, a potential sink for these elements is removed, and this is reflected in the higher concentration of these elements in the leaves. It is significant that the data in figures 6 and 7 indicate that the developing fruit is able to effect a decrease in the potassium and phosphorus content of even the youngest leaves. The mineral requirements of the developing fruit are satisfied at the expense of the vegetative tissues, if the external supply falls short of satisfying the needs of both (4). A comparison is suggested with the die-back condition of the prune, in which a severe leaf scorch is associated with low potassium content of the leaves (at least late in the summer) and a heavy crop of fruit. The condition was prevented by removing the crop by hand early in the season (8).

Defloration of plants grown in a complete nutrient solution did not result, within the sampling period, in marked increase of potassium, calcium, magnesium, or phosphorus in the vegetative tissues. It is possible that defloration, by removing a physiological sink, has impeded salt absorption in a manner analogous to the decreased salt absorption observed in "high-salt" plants (7). The suggestion is that the morphological effects of defloration (figs. 2, 3, 6) may be primarily associated with shifts in the organic constituents.

Attention has already been drawn to the accumulation of starch, sugars, and organic nitrogen in the deflowered plants. It is probable, of course, that even more striking changes were brought about by defloration in some other organic constituents not included in this investigation. Organic acids, and particularly growth substances or florigens, would seem to offer likely subjects for study. The unusual growth proliferation on the leaves and the occurrence of rootlike structures on the deflowered stems resemble those frequently produced by the application of hormone-like substances. The withering of the terminal growing point on the deflowered plants points to the likelihood of some toxic accumulation in this region of one or more organic constituents.

The morphological observations and chemical data leave no doubt that flowering and fruit development in a healthy tomato plant is a normal activity, which, if not permitted to proceed, results in important metabolic derangements. The unusual growth effects found on the deflowered plants may perhaps be regarded as an attempt on the part of the plants to make use, under the stimulus of growth substances, of the accumulated organic reserves which would normally lead to flowering and fruiting. As mentioned previously, the other outlet initiated by the plant for the utilization of these reserves, as reflected in the emergence of new growing points from the leaf axils, was also blocked by manual removal of the new growth.

The evidence here presented offers little encouragement to attempts to improve, from the standpoint of human nutrition, the mineral composition, with respect to calcium, of tomato or probably of other fruits by liberal soil fertilization measures. The tomato is not a rich source of calcium in the human diet, and even if marked increase in the calcium content of the fruit could be effected, it would not alter our dependence on other important sources of calcium, especially milk and cheese.

The prevalence of symptoms resembling blossom-end rot in the fruit from the phosphorus-deficient plants adds another observation to the etiology of this

disturbance. It seems possible that blossom-end rot may be associated with a number of nutritional causes all resulting in withdrawal of water from the blossom end of the fruit and subsequent breakdown of tissues.

The influence of nutrient conditions on the storage quality of tomatoes was investigated by MacGillivray (10).

Summary

1. Tomato plants were grown through the fruiting stage under greenhouse conditions in soil, sand, and large-scale water cultures, with nutrient solutions of varying composition.

2. The composition of the fruit with respect to constituents derived through absorption by roots was, in contrast to the modifiable composition of foliage, on the whole little affected by the kind of nutrient medium in which the plants grew. The composition was also not found to be materially altered in water-culture experiments in which the total concentration and relative proportions of nutrients were varied. Marked reduction was found in the potassium or phosphorus content of fruit developed on plants grown in a potassium- or phosphorus-deficient nutrient solution.

3. On a percentage dry-weight basis the concentration of potassium, calcium, magnesium, phosphorus, and nitrogen was markedly lower in the fruit than in the leaf, and the relative proportions of these nutrients were strikingly altered.

4. The manual removal of flowers in plants grown in a complete nutrient solution resulted in distinct alterations in growth, shown by thickening of stems and leaves, proliferation of new growing points at the leaf axils, and emergence on the stem of rootlike structures. Defloration was found to be associated with marked accumulation of starch in all tissues and of sugars and organic nitrogen in the stems and petioles. However, defloration was not found to influence materially the composition of foliage with respect to phosphorus, calcium, or magnesium. The potassium content of the petioles was higher in the fruitful plants, but no marked difference was found in the potassium content of leaf blades.

5. Defloration of plants kept in a potassium-deficient nutrient solution delayed the onset of potassium-deficiency symptoms and was associated with a higher potassium content in the young leaves of the deflowered than in the fruitful plants. The otherwise low potassium content of leaves showed an increasing upward gradient as contrasted with a downward gradient in plants grown in a complete nutrient solution. Downward calcium and magnesium gradients found in plants grown in complete nutrient were also found in leaves of potassium-deficient plants, but the latter had a higher content of these elements in the fruitful than in the deflowered plants.

6. Defloration of plants kept in a phosphorus-deficient nutrient solution delayed the onset of phosphorus-deficiency symptoms and was associated with a higher phosphorus content in the young leaves of the deflowered plants. The otherwise low phosphorus content of leaves from all phosphorus-deficient plants, whether fruitful or deflowered, showed—in contrast with the control plants—increasing upward gradient.

7. Several general considerations are given on the relation of the external nutrient supply and fruiting to the distribution of nutrients within the plant.

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EFFECTS OF NUTRIENT-SOLUTION CONCENTRATION ON THE GROWTH OF MARCHANTIA POLYMORPHA[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 552

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Introduction

Following forest fires, and even burns of local extent, *Marchantia polymorpha* commonly invades the bare area, forming an almost pure stand (**6**, and others). Dry heat is known to make available greater quantities of solutes, which may inhibit green plants such as oats but stimulate the growth of *Pyronema*, a fungus (**23**). Since little is known concerning the concentration of a complete nutrient solution which would be optimum for a liverwort such as *Marchantia*, the following experiments were performed.

In tests conducted as preliminary to those already reported (**31**, **32**), it was established that a complete nutrient solution with an osmotic concentration of about 0.75–1.00 atmospheres, as used in greenhouse culture of common vegetable plants, was not conducive to maximum growth of *Marchantia*. At that time (1939) it was estimated that a dilution of approximately one-fifth was a more desirable solution. The present report is apparently the first to determine the dilution which is optimum for the growth of this species of *Marchantia*. Since the relative concentrations of the common ions had already been determined in this laboratory, a series of solutions were devised in which the relative proportions of the various salts remained constant but the amount varied according to a definite plan.

The problem of varying the concentration of a balanced or complete nutrient solution, with consequent changes in osmotic concentration, diffusion rates, and total salts available has been considered for many years. Experiments concerned with varying the amount of one or two of the constituents of a nutrient solution usually introduce several additional problems (**15**). Results when the amount of salts is changed proportionately must be judged by the range of concentration employed, the kind of plant, the season of the trials, the substrate used (whether sand or liquid culture), and other factors. A summary of such investigations has been made by TOTTINGHAM (**29**). LIVINGSTON (**15**), SHIVE (**24**), and others have reported earlier experiments dealing with the effect of varying the concentration of all salts. In general, it was found that the weaker or intermediate nutrient solutions produced plants superior in size or dry weight (**1**, 713.4 p.p.m.; **2**, 0.3 per cent "normal" solution; **3**, 300 p.p.m. in water culture, 2500 p.p.m. in sand; **9**, 1.5 atmospheres of pressure; **10**, 0.6 atmospheres or less; **20**, 1/50 dilution of Knop's solution; **22**, 2880 p.p.m.; **25**, 1.75 atmospheres; and **27**, salt concentration approximately equal to solution 5 of present report). Plants utilized for these experiments were tobacco, oats, wheat, tomato, barley, duckweeds, buckwheat, and pineapple, with the cereal grains predominating. On the other hand, several workers maintain that the solutions with higher salt content produced optimum growth (**4**, 3000 p.p.m.; **7**, **8**, 1142 p.p.m.; **19**, 2200 p.p.m.; and **28**, all utilizing water cultures). It is noted that solutions considered concentrated by some are classed as intermediate by others. A few workers favor the view that the concentrations of nutritive salts can

[1] This work was supported in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

vary widely before any noticeable effect is demonstrated (**11**, 1433–5337 p.p.m.) or that such fluctuations are relatively unimportant (**13**). In several of the investigations just mentioned solutions leached from the soil were employed (**7**, **8**, **22**).

While dry weights of plants or plant parts and similar data are important in the determination of the optimum solution, anatomical investigations often supplement or even change the interpretation of data. LIVINGSTON (**15**) noted that the usual branched filament of the alga *Stigeoclonium* was supplanted by spherical colonies of cells when strong solutions of inorganic salts were used as a growing medium. SCHOENE (**21**) demonstrated that no rhizoids are produced on newly-formed protonemata of *Funaria* and other mosses. The most critical studies of NIGHTINGALE and FARNHAM (**16**) established that dilute nutrient solutions resulted in vigorously growing, succulent sweet-pea plants. The failure of cells to mature rapidly, especially at the base of the pedicel, was responsible for the formation of an abscission layer and subsequent loss of the flower. HAYWARD and LONG (**9**) demonstrated that in tomato high nutrient concentrations favored rapid maturation, especially of vascular tissues, with consequent smaller cells and organs.

A correlation between gross appearance, weights, area, and anatomical changes of *Marchantia* is the object of the experiments reported here.

Material and methods

Clones of male and female plants of *Marchantia polymorpha*, designated cultures A and B, respectively (**32**), were again used in these experiments. The culture method described previously (**31**) consisted of placing six plants on a disk of glass cloth supported by a glass rack and placed in the open half of a large moist chamber. Plants and apparatus were pressure-rinsed with distilled water daily, after which they were supplied with 150 cc. of the appropriate nutrient solution. The present investigation is designated experiment 11 and was conducted in the greenhouse from March 29 to April 25–26, 1941 (27 days). A photoperiod of 17 hours was provided by supplementing daylight with 200-watt Mazda filament bulbs in reflectors (**32**). The bulbs were automatically lighted from 6:00 P.M. to midnight.

The basic solution was determined by means of nutrient triangle experiments and was suggested in a previous paper (**32**, p. 325). This solution is designated number 5 in table 1. By using higher as well as lower relative proportions of the fundamental solution (number 5), ten solutions were prepared with the extremes of concentrations ranging from 160 to 1. Solution 2 is of approximately the same concentration as nutrient solutions used to grow greenhouse plants such as beans, tomatoes, etc. Solution 1 is twice as concentrated as solution 2; and beginning with solution 6, succeeding dilutions are by one-half.

Osmotic concentrations were determined cryoscopically, using freshly prepared solutions. The hydrogen-ion concentrations were made with an instrument equipped with a glass electrode. The higher dilutions tended to be slightly more acid than less dilute ones, probably because of the acidity of the distilled water. When left standing for several weeks in covered, glazed earthenware containers, the solutions tended to become more alkaline, with proportionally larger increases as dilutions were greater.

The molar concentration of solution 5 is: K 0.0012, Ca 0.0007, Mg 0.0014, NO_3

0.0034, PO_4 0.0004, and SO_4 0.0008 mols per liter. These ten solutions did not remain stable when plants were actively growing on them. Owing to the high nitrate content and the low phosphate concentration, the hydrogen-ion concentration decreased rapidly soon after the solution was supplied to the plant. In several stock cultures, readings of pH 10.0 or greater were recorded without pronounced detrimental effects to the plants. This tendency toward alkalinity is well known (30). When a solution can be used in which KH_2PO_4 or other phosphate salts (17) are relatively high, and even greater in amount than any other salt (26), this fluctuation in pH is greatly diminished by the buffer action of the phosphate.

Investigation

Weight, area, and gemmae cups

Each *Marchantia* cutting, consisting of one apical notch and surrounding tissue, averaged 3 sq. cm. in area when planted on March 29. Each group of six plants received daily portions of 150 cc. of solution 5 for the first 3 days, since it was known that this nutrient combination promoted rhizoid formation. It has been used successfully for several years in growing the stock plants. Following this preliminary treatment, the specified variations of this solution (table 1) were supplied each culture of six plants again at a rate of 150 cc. per day.

After about the tenth day, plants supplied with solutions 1 and 2 became

TABLE 1

QUALITIES OF NUTRIENT SOLUTION EMPLOYED IN EXPERIMENT 11 (MARCH–APRIL, 1941)

Solution no.	Relative concentration of solutions		Molar concentration of salts (millimols)					Concentration of micronutrients (p.p.m.)	Osmotic concentration in atmospheres (average)	pH (average)
	Decimal	Fractional	KNO_3	$Ca(NO_3)_2 \cdot 4H_2O$	$Mg(NO_3)_2 \cdot 6H_2O$	KH_2PO_4	$MgSO_4 \cdot 7H_2O$			
1....	1.00	$\frac{100}{100}$	8.0	7.0	6.0	4.0	8.0	2.0*	1.83	4.93
2....	0.50	$\frac{50}{100}$	4.0	3.5	3.0	2.0	4.0	1.0	0.94	5.14
3....	0.30	$\frac{48}{160}$	2.4	2.1	1.8	1.2	2.4	0.6	0.59	5.38
4....	0.20	$\frac{32}{160}$	1.6	1.4	1.2	0.8	1.6	0.4	0.37	5.66
5....	0.10	$\frac{16}{160}$	0.8	0.7	0.6	0.4	0.8	0.2	0.21	5.94
6....	0.075	$\frac{12}{160}$	0.6	0.525	0.45	0.3	0.6	0.15	0.18	5.92
7....	0.050	$\frac{8}{160}$	0.4	0.350	0.30	0.2	0.4	0.10	0.13	6.05
8....	0.025	$\frac{4}{160}$	0.2	0.175	0.15	0.1	0.2	0.05	0.08	6.03
9....	0.0125	$\frac{2}{160}$	0.1	0.0875	0.075	0.05	0.1	0.025	0.05	6.09
10....	0.00625	$\frac{1}{160}$	0.05	0.04375	0.0375	0.025	0.05	0.0125	0.01	5.95

* Concentration of $MnSO_4$, $ZnCl_2$, and $Na_2B_4O_7$, singly; concentration of $FeSO_4$ is 0.1 of the figure given.

slightly translucent and were definitely smaller than plants on more dilute solutions. Plants on solutions 9 and 10 gradually developed red-purple scales, rhizoids, and ventral epidermis after a similar period. The development of translucency in plants grown on nutrient solutions of high concentration and the anthocyanin coloration of plants supplied with solutions of lesser salt content continued until the end of the experiment. Plants growing on solutions containing intermediate amounts of salts were generally the light to dark green color characteristic of *Marchantia*. The color and the general aspect of the plants at the end of the experiment may be summarized as follows:

SOLUTION 1.—Thalli were watery; wings and even tips of many plants were dead. Tips of surviving plants grew into elongated thalli, resembling regenerated tips of overwintered or injured plants commonly seen in discarded stock cultures.

SOLUTION 2.—Low-lying plants were watery, others were bright yellow-green. Tips generally were broad, and all plants were alive.

SOLUTIONS 3, 4, 5.—With decreasing salt content the plants tended to increase in size, to become darker in color, to possess ascending tips, and to develop rhizoids more abundantly.

SOLUTIONS 6, 7, 8, 9.—The plants became increasingly darker red-purple (rhizoids, scales, and lower epidermis) and decreased slightly in size with decreased concentration of the solution. Rhizoids were produced in increasing quantities, and thalli were brash.

SOLUTION 10.—The excessive development of anthocyanin on the ventral surface and the yellow-green of the dorsal surface indicated that the lack of an adequate nitrate supply was more pronounced than the lack of phosphate. The smaller number of gemmae cups and the narrow thallus with few dichotomies also expressed nitrate more than phosphate deficiency. These plants much resembled those of previous experiments in which nitrates and phosphates were lacking (31).

Table 2 shows the various trends of growth. Fresh and dry weights tend to increase progressively from solutions 1 to 6 and to decrease from solutions 7 to 10. Plants grown on solution 5 had a total fresh weight of 18×8.518 gm., which was slightly higher than the weight of plants on solution 6 (18×8.096 gm.). The greater weight of plants on solution 5 in this instance may be attributed to the very favorable growth of all female plants on the latter solution. The fresh weights of the male plants and the calculated dry weights of both sexes were all greater when plants were growing on solution 6. When actual dry weights of plants growing on the two solutions are known and compared, both sexes produced more substance when grown on solution 5. Male plants in one culture dish supplied with solution 5 grew poorly, which, at least in part, accounts for the failure of solution 5 to be optimum by all methods of calculation.

In area, the female plants on solution 5 exceeded similar plants on solution 6. In all other comparisons of area, solution 6 is optimum to solution 5. As shown in table 2, male plants on solution 4 were slightly larger on the average than similar ones on solution 5 but did not approach the average area of male plants on solution 6.

Gemmae cups vary greatly from plant to plant, but a comparison of the totals of averages based on eighteen plants (table 2) indicates that the maximum number was produced on solution 4. For both

TABLE 2

GROWTH OF MARCHANTIA POLYMORPHA ON TEN NUTRIENT SOLUTIONS IN WHICH AMOUNTS OF CONSTITUENT SALTS VARIED PROPORTIONATELY (TABLE 1). FIGURES ARE AVERAGE FOR ONE PLANT, BASED ON EIGHTEEN PLANTS GROWN IN THREE CULTURE DISHES. MAXIMUM VARIATION IS GIVEN

Solution no. and sex	Weight (gm.)			Area (sq. cm.) (original area 3 sq. cm.)	No. of gemmae cups	Ratio: weight (mg.) / area (sq. cm.)	
	Fresh	Dry					
		Calculated	Weighed			Dry‡	Fresh
1 ♂	0.849 (±0.177)	0.0543 (±0.0032)	.....*	16.11 (±6.11)	7.11 (± 5.89)†	3.89	53.20
1 ♂	0.729 (±0.099)	0.0585 (±0.0073)	0.0611 (±0.0049)	13.55 (±5.45)	6.67 (± 3.33)		
1 ♀	0.699 (±0.093)	0.0452 (±0.0113)	.....	14.17 (±2.17)	2.44 (± 9.56)	3.84	54.60
1 ♀	0.724 (±0.059)	0.0564 (±0.0144)	0.0550 (±0.0145)	11.89 (±4.28)	2.50 (± 6.50)		
2 ♂	1.359 (±0.186)	0.0835 (±0.0187)	.....	22.89 (±4.56)	9.83 (±11.17)	4.09	63.49
2 ♂	1.502 (±0.539)	0.1017 (±0.0326)	0.1009 (±0.0328)	22.17 (±9.84)	13.72 (± 7.72)		
2 ♀	1.507 (±0.272)	0.0861 (±0.0139)	.....	25.33 (±2.84)	7.94 (±10.06)	3.72	64.92
2 ♀	1.461 (±0.603)	0.0852 (±0.0272)	0.0841 (±0.0255)	20.39 (±8.56)	9.78 (±13.22)		
3 ♂	1.768 (±0.155)	0.0954 (±0.0064)	.....	29.78 (±0.95)	21.50 (± 9.50)	3.59	63.03
3 ♂	2.059 (±0.431)	0.1231 (±0.0124)	0.1226 (±0.0137)	30.94 (±6.39)	22.33 (±13.33)		
3 ♀	1.827 (±0.552)	0.0939 (±0.0247)	.....	30.06 (±6.56)	16.61 (±18.39)	3.37	64.04
3 ♀	1.845 (±0.750)	0.0999 (±0.0416)	0.0995 (±0.0402)	27.28 (±8.95)	14.44 (±14.44)		
4 ♂	1.708 (±0.460)	0.0927 (±0.0214)	.....	32.83 (±5.83)	20.22 (±14.78)	3.32	55.44
4 ♂	2.114 (±0.445)	0.1355 (±0.0333)	0.1365 (±0.0318)	36.11 (±0.61)	24.22 (±13.78)		
4 ♀	2.147 (±0.216)	0.1188 (±0.0137)	.....	37.44 (±2.11)	21.33 (±16.67)	3.24	61.65
4 ♀	2.473 (±0.120)	0.1237 (±0.0179)	0.1241 (±0.0154)	37.50 (±2.00)	17.72 (±15.72)		
5 ♂	1.678 (±0.525)	0.1046 (±0.0296)	.....	35.94 (±5.77)	20.22 (±13.22)	3.30	54.53
5 ♂	1.996 (±0.484)	0.1164 (±0.0204)	0.1177 (±0.0196)	31.44 (±4.56)	18.56 (± 7.56)		
5 ♀	2.176 (±0.473)	0.1244 (±0.0052)	.....	39.44 (±2.44)	15.27 (± 9.27)	3.32	60.98
5 ♀	2.668 (±0.662)	0.1458 (±0.0028)	0.1391 (±0.0131)	40.00 (±3.00)	11.11 (±11.89)		
6 ♂	2.042 (±0.211)	0.1185 (±0.0107)	.....	39.33 (±2.83)	20.67 (±12.33)	3.28	55.10
6 ♂	1.809 (±0.186)	0.1156 (±0.0261)	0.1109 (±0.0333)	30.56 (±4.44)	15.39 (±12.39)		
6 ♀	2.157 (±0.270)	0.1452 (±0.0300)	.....	40.94 (±2.23)	9.94 (± 7.94)	3.54	54.35
6 ♀	2.088 (±0.282)	0.1302 (±0.0110)	0.1314 (±0.0124)	37.17 (±3.17)	7.67 (± 7.67)		
7 ♂	1.955 (±0.077)	0.1088 (±0.0072)	.....	36.06 (±1.44)	18.11 (±12.11)	3.48	58.15
7 ♂	1.999 (±0.178)	0.1277 (±0.0167)	0.1280 (±0.0165)	31.94 (±1.39)	16.78 (± 7.78)		
7 ♀	2.272 (±0.074)	0.1232 (±0.0111)	.....	40.11 (±1.06)	6.89 (± 8.11)	3.30	57.36
7 ♀	2.052 (±0.521)	0.1262 (±0.0200)	0.1253 (±0.0183)	35.28 (±6.11)	4.11 (± 6.89)		
8 ♂	1.809 (±0.108)	0.0981 (±0.0096)	.....	34.00 (±3.17)	16.00 (±12.00)	3.18	53.76
8 ♂	1.533 (±0.132)	0.1027 (±0.0090)	0.0996 (±0.0076)	28.17 (±1.67)	13.22 (±13.78)		
8 ♀	2.027 (±0.352)	0.0964 (±0.0046)	.....	34.06 (±1.27)	5.72 (± 8.28)	3.05	56.82
8 ♀	1.976 (±0.193)	0.1184 (±0.0098)	0.1182 (±0.0101)	36.39 (±3.89)	4.06 (±10.94)		
9 ♂	1.659 (±0.122)	0.0783 (±0.0052)	.....	30.94 (±2.06)	10.11 (± 7.89)	2.97	49.98
9 ♂	1.334 (±0.076)	0.0988 (±0.0087)	0.0994 (±0.0084)	28.94 (±3.06)	11.94 (± 7.94)		
9 ♀	1.837 (±0.435)	0.0916 (±0.0031)	.....	34.78 (±0.89)	5.17 (± 8.83)	3.15	51.94
9 ♀	1.464 (±0.279)	0.1106 (±0.0071)	0.1089 (±0.0048)	28.78 (±1.22)	3.89 (± 7.11)		
10 ♂	1.367 (±0.328)	0.0800 (±0.0117)	.....	30.61 (±0.56)	5.89 (± 8.11)	3.03	51.21
10 ♂	1.543 (±0.490)	0.0913 (±0.0093)	0.0923 (±0.0095)	26.22 (±3.05)	8.61 (± 7.39)		
10 ♀	1.547 (±0.089)	0.0832 (±0.0173)	.....	29.89 (±0.56)	1.33 (± 4.67)	3.07	52.75
10 ♀	1.422 (±0.129)	0.0893 (±0.0112)	0.0896 (±0.0111)	26.39 (±3.39)	2.06 (± 4.94)		

*One plant from each of three culture dishes of each nutrient solution was preserved for anatomical study, hence no direct weighing of all dry plants in such lots could be made.

† Maximum variations are based on actual counts of gemmae cups on each plant.

‡ When available, the actual dry weight is utilized; otherwise the calculated dry weight is used.

sexes the numbers are greater for plants growing on solution 3 than on solution 5, but both treatments produced fewer gemmae cups than plants on solution 4. In all instances where totals from eighteen or more plants are compared for number of gemmae cups, the male plants of each treatment produced a greater number of these reproductive bodies than the female ones. In this experiment a total of 3060 gemmae cups was counted on female and 5420 on male plants. This ratio of 1 to 1.44 is less than the ratios of 1 to 4 and 1 to 6.6 reported for experiments 8 and 9 and experiment 10 (**31, 32**), respectively, where solutions approximated number 5 of this experiment with respect to osmotic concentration.

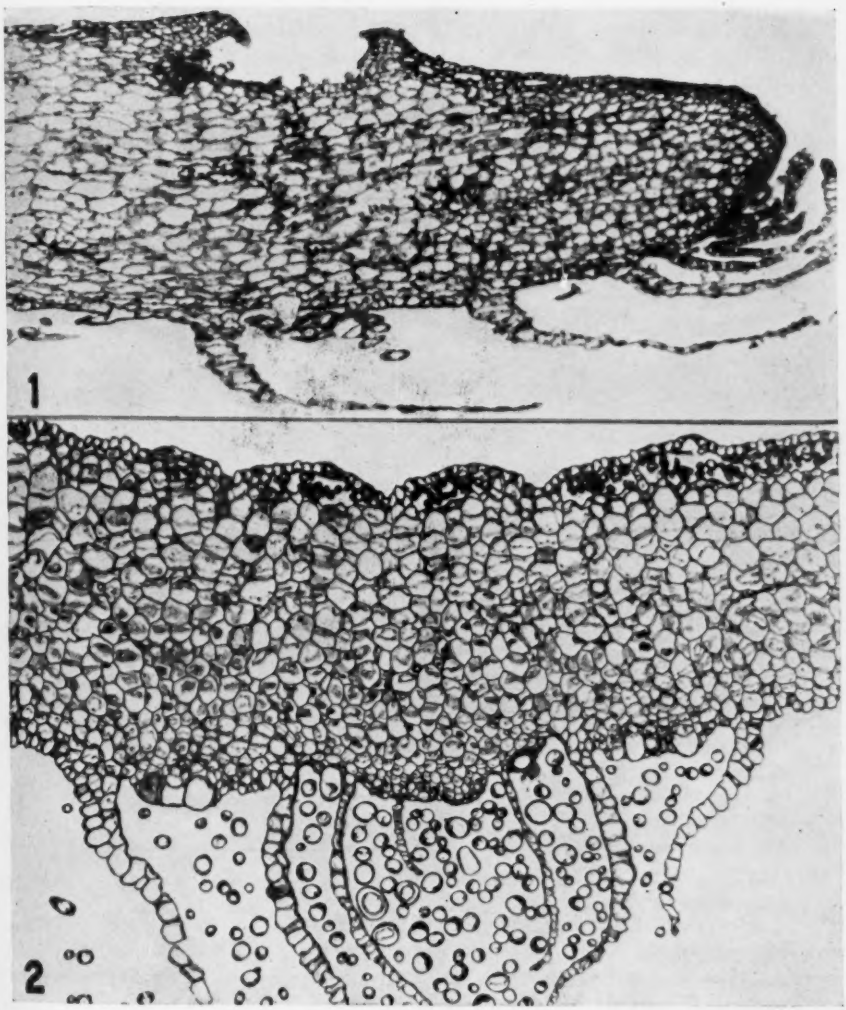
The ratios of the area to the weight, based on the averages of the plants of each sex, varied only moderately, with a tendency toward a higher ratio in plants grown on more concentrated solutions. The larger ratios (4.09 on the dry-, 64.92 on the fresh-weight basis) were found in plants of treatment 2, indicating a relatively smaller area and a relatively larger weight compared with plants growing on other treatments. Smallest ratios were secured for plants growing on treatments 8, 9, and 10.

The ability of *Marchantia* to survive great changes in hydrogen-ion concentration is evidently shared by many mosses (**12**). Suitable buffers containing no phosphate (because of increased growth on solutions low in phosphates) should be added to nutrient solutions, thus controlling a factor which has not received enough attention in the experiments reported here.

Anatomical variations

Cross sections of the thallus, approximately 2 mm. back from the apical notch, and longitudinal sections through the notch, were mounted and stained by the usual procedure. Sections were 7μ in thickness. Sixty plants were investigated in this manner. Plants on concentrated nutrient solutions possessed thin cell walls which collapsed to a certain degree when preserved in formalin-acetic acid-alcohol. Figure 1 is a longitudinal section of a male plant on solution 1, passing through a gemma cup located dorsally. The apical cell is at the extreme right, just above the youngest scales. Ventrally, rhizoids and scales are distributed normally, although cells of the latter are collapsed. A very small zone of growth surrounding the apical cell contrasts with the remainder of the thallus, which has matured rapidly—as indicated by the optically empty cells. Many internal necrotic areas characterize this section and nearly all other plants growing on solution 1. The lack of gemmae in the cup and photosynthetic filaments in the air chambers is striking.

The other extreme of thallus development is shown in figure 2, a cross section of a female plant in the midrib region which was supplied with solution 10. It is characterized by optimum development of rhizoids of the smooth and tuberculate varieties, scales, storage region, photosynthetic filaments, and pores in the upper epidermis. Most cells have contents, and cell walls are unusually sturdy. No collapse of any cell is discernible. Dassonville (**5**) reported that seed plants grown in distilled water possessed a more prominent cuticle and fragile cell walls. Tissues matured and lignified rapidly and early, and secondary growth was limited. In contrast, *Marchantia* gametophytes develop thicker walls when only a very small amount of salt is present in the solution. Rapid and early maturity of tissues when plants are supplied concentrated solutions is equal-



FIGS. 1, 2.—Sections through thallus of *Marchantia* near growing tip: Fig. 1, longisection of male plant on solution 1 showing necrotic areas and lack of living gemmae. Fig. 2, cross section of female plant on solution 10 showing thickened cell walls and well-developed photosynthetic filaments.

ly true in *Marchantia*, sweet pea (**16**), and tomato (**9**).

Solutions between the extremes of 1 and 10 permitted development in a manner intermediate between that of the two plants illustrated. No necrotic areas were found in plants on solutions other than 1 and 2. As solutions became progressively more dilute, the collapse of cells lessened as cell walls increased in thickness. Gemmae production in solution 2 was much greater than in the most concentrated solution, and the number of cups reached a maximum in plants on solution 3. A survey of all microscopic sections in this experiment, as well as those of previous ones, indicates that—regardless of great variations in individual nutrient salts and variations in total salt content—*Marchantia* thalli produce a storage tissue 14–20 cells in thickness.

The extreme of cell derangement is shown in figure 3, where the optically empty cells of the older part of the thallus contrast sharply with the zones of necrotic cells nearer the apex.

Discussion

Marchantia polymorpha makes the greatest vegetative growth on solutions which have an osmotic concentration ranging from 0.21 to 0.18 atmospheres (solutions 5 and 6). That other plants grow larger on dilute solutions has been reported by SAEGER (**20**), who found optimum growth of duckweeds on a 1/10 dilution of Knop's solution, and by LIEBIG (**14**), who grew *Isoetes* from spores on diluted Knop's solution. That protonema of *Funaria* and *Ceratodon* can withstand solutions of high osmotic value is reported by PRINGSHEIM and PRINGS-

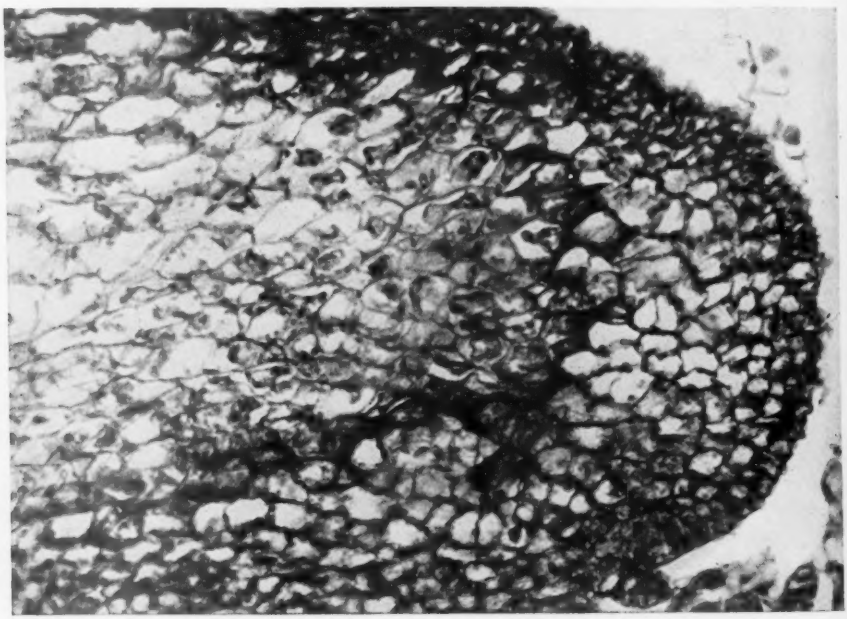


FIG. 3.—Longisection of male plant on solution 2 showing clear cells in center of thallus and concentric zone of necrotic cells. Apical cell at lower right.

HEIM (18) and is evident from the report of SCHOENE (21). The exact conditions which *Marchantia* encounters when invading a newly-burned area has not been determined. From greenhouse experiments it is concluded that while this liverwort is hardy in extreme concentrations of nutrient solutions, its optimum vegetative development is in the dilute medium. The enormous size of some *Marchantia* plants in mountainous regions may depend on the attainment of such a dilution.

Solution 3, with an osmotic concentration of about 0.59 atmospheres, was most conducive to the development of gemmae cups (table 2). The larger number of cups on plants supplied with concentrated solutions was noted several years ago in preliminary experiments. Development of the cups evidently is dependent upon several factors when results of this and previous experiments are summarized. Four conditions contribute to greater production: (1) Relatively high osmotic concentration of the nutrient solution (table 2) results in the formation of a greater number of these reproductive bodies, especially when the smaller size of such plants is considered. (2) When phosphates are eliminated from the nutrient solutions and nitrates are abundantly supplied, a large number of cups are produced by relatively small plants (31, 32). (3) Male plants of clone A used for these experiments exceed female plants of clone B in production of the cups (table 2, and previous experiments, 31, 32). (4) More gemmae cups are produced on a short photoperiod of about 8 hours than on a long photoperiod of 17–18 hours (31).

If a common denominator exists for these factors it has not yet been determined for this plant.

Preserving one from each culture of six plants in previous experiments and from half the cultures in this one necessitated the selection of a representative plant for anatomical study and the calculation of the dry weight of six plants from the percentage dry weight of five plants and the known fresh weight of all. This method had not been checked for validity in the present series of experiments until experiment 11 permitted the culture of enough plants to make direct comparison possible. At harvest time half the cultures were treated as if one plant from each was to be preserved in an alcoholic solution. Instead it was segregated and its weight compared directly with the calculated weight (table 2). The greatest discrepancies between actual and calculated weights appeared in the averages of plants 5♀ and 6♂, with differences of 6.7 and 4.7 mg. per plant, respectively. In nearly all other cultures the dry-weight differences were only a milligram or less. About half the actual weights were higher and the other half lower than the calculated ones. With greater care in weighing fresh plants these differences should become even less.

Summary

1. Using methods previously described, cultures of two clones of *Marchantia polymorpha* were supplied with ten nutrient solutions ranging in concentration from 1.83 to 0.01 atmospheres, of which solution 5, with a concentration of 0.21 atmospheres and 0.0033 mols per liter, was optimum for vegetative growth —as judged by fresh and dry weights and area. It contained 1.6 cc. KNO_3, 1.4 cc. $Ca(NO_3)_2$, 1.2 cc. $Mg(NO_3)_2$, 0.8 cc. KH_2PO_4, and 1.6 cc. $MgSO_4$, each of a 0.5 *M* solution per liter, and the usual micro-nutrients. Solution 6 was by some standards slightly superior and by others inferior to solution 5. Its concen-

tration was 75 per cent that of solution 5. The pH characteristics of these solutions are briefly discussed.

2. Gemmae cups are most numerous on plants grown on solution 4.

3. Gemmae cups are produced on female and male plants in a ratio of 1 to 1.44—a lower ratio than in previous experiments.

4. Solutions with high salt concentration tended to kill the growing tips of plants and produced translucent thalli. Maturity was rapid, with cells slightly smaller than those grown on dilute solutions. Solutions low in salts favored the development of anthocyanin, numerous rhizoids, and a sturdy plant body possessing cells with living contents and thicker cell walls.

5. The calculation of dry weights of six plants from the weight of five and the fresh weights of six and five plants is reasonably accurate.

UNIVERSITY OF CHICAGO

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CHROMOSOME NUMBERS IN THE SUBFAMILY AURANTIOIDEAE WITH SPECIAL REFERENCE TO THE GENUS CITRUS[1]

C. A. KRUG[2]

Introduction

The orange subfamily, Aurantioideae, constitutes one of the important subdivisions of the Rutaceae, comprising, according to SWINGLE (**26**), who proposed a new taxonomic arrangement of this group, a total of 2 tribes, 6 subtribes, 33 genera, 201 species, and 15 varieties. It includes types which show a wide range of morphological and physiological characters, varying from xerophytic forms, as *Eremocitrus glauca* (Lindl.) Swingle, growing wild in the Australian deserts, with small, narrow, and linear leaves, to forms well adapted to humid tropical zones, as the pummelos or shaddocks (*C. grandis* Osbeck), natives of Malaysia and Polynesia; some have simple leaves with wings of varying sizes on their petioles, as *Citrus;* others have evergreen compound ones, as *Feroniella lucida* Swingle; and still others have trifoliate and deciduous ones, as *Aegle marmelos* Correa; the fruits vary from little berries, as in *Murraea exotica* L., to large hesperidia, as the well-known pummelos (*Citrus grandis* Osbeck), weighing several pounds each.

Considering the great diversity of characters in this group, considerable variation in its cytological constitution might also be expected, especially in regard to chromosome number. On the contrary, this number has been found relatively constant for all members of the subfamily so far investigated, the basic number for all of them being n = 9 and nearly all cultivated varieties being diploid (2n = 18).

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STRASBURGER (**25**) was apparently the first to make cytological investigations on one of the members of this group, the genus *Citrus*, mentioning 8 as the haploid number; also OSAWA (**22**) gives this haploid number as the most probable for *Citrus*. All other workers, particularly LONGLEY (**15, 16**), FROST (**3–8**), and NAKAMURA (**18–20**), agree that 9 is the basic number. STRASBURGER'S and OSAWA'S failure to determine the correct number probably was due to defective technique. With respect to chromosome morphology, little information is available. KANDELAKI (**10**), investigating several species of *Citrus* and *Fortunella margarita* (Lour.) Swingle, attempted to classify the metaphase chromosomes of somatic mitosis into three groups: one with distinctly unequal-armed chromosomes, another with only slightly unequal-armed chromosomes, and a third group consisting of chromosomes with satellites. From the findings of several other investigators, including the writer, it seems unquestionable that marked size differences exist between chromosomes of the same somatic set in most of the species and genera of this subfamily so far studied.

In the present paper the chromosome numbers of several genera, species, and varieties of the Aurantioideae not previously determined are presented, and table 1 includes all determinations made so far that were found in the literature available to the writer.

Material and methods

The following determinations were made on material collected at the University of California Citrus Experiment Station, Riverside, California, in the

spring of 1941. For correct identification of this material (excluding horticultural varieties) the writer is especially indebted to Dr. W. T. SWINGLE, to whom herbarium material was submitted in Washington, D.C. Chromosome counts were made in root tips from leafy cuttings which were rooted in sand in the greenhouse; roots were fixed in Craf (**23**) and dehydrated by the butyl-alcohol method. Paraffin sections were stained with Heidenhain's haematoxylin. A few determinations were also made in aceto-carmine smears of young anthers.

NON-CITRUS MATERIAL.—Chromosome counts were made in the following material:

1. *Clausena lansium* (Lour.) Skeels. (C.E S. 1460, 7A–7–4; C.P.B. 2904).[3] This genus, native of South China, belongs to the tribe Clauseneae, subtribe Clauseninae; it is represented by large trees with pinnately compound leaves; its flowers are white and borne in large panicles, the fruits being small round berries. 2n = 18 (fig. 1).

2. *Atalantia citroides* Pierre ex Guill. (C.E.S. 1430, 7A–7–9; C.P.B. 7534). According to SWINGLE, the material investigated might possibly belong to *A. monophylla*, as unfortunately no flowers were available for specific identification. *Atalantia* is one of the genera of the tribe Citreae, subtribe Citrinae, being characterized by short, narrow, and thick leaves. 2n = 18 (fig. 2).

3. *Microcitrus* spp. (C.E.S. 1484, 7A–12–2; C.E.S. 1485, 7A–12–3; C.E.S. ?, 7A–14–4). Three species were examined at the Citrus Experiment Station, two of which were labeled respectively *M. australasica* var. *sanguinea* and *M. virgata*. The validity of these specific determinations could not be established. All three species have small, blunt, ovate leaves and sharp spines, characteristic of this genus. 2n = 18 (figs. 3, 4).

4. *Afraegle gabonensis* (Swingle) Engler (C.E.S. 1432, 7A–8–8; C.P.B. 7516–A). The genus belongs to the tribe Citreae, subtribe Balsamocitrinae. The Riverside specimen is a small tree with *Citrus*-like leaves, which are somewhat purplish in color; its thorns are long and stout; it does not seem to flower and fruit in California. 2n = 18.

CITRUS MATERIAL.—The following species and varieties of *Citrus*, economically the most important group, were also found to have 2n = 18:

1. *Citrus aurantifolia* Swingle: *Kusai lime[4] (C.E.S. 452, 3A–15–3); Mexican lime—considered the type species—(C.E.S. 1710, 3A–14–3) (fig. 5); Palestine Sweet lime (C.E.S. 1482, S1A–6–13; C.P.B. 1158).

2. *C. celebica* Koord. var. *southwickii* (Wester) Swingle (C.E.S. 2453, 3A–36–2; C.P.B. 10125) (fig. 6).

3. *C. hystrix* DC. (C.E.S. 2454, 3A–37–1; C.P.B. 2881).

4. *C. ichangensis* Swingle (C.E.S. 2431, S1A–10–15; C.P.B. 954) (fig. 7).

5. **C. leiocarpa* Hort. nov. var. (possibly a form of *C. tachibana* [Mak.] Tan.) (C.E.S. 2448, 7A–8–6; C.P.B. 10280) (fig. 8).

6. *C. limon* (L.) Burm.: Lisbon lemon (C.E.S. 584, S1A–4–4); *Meyer lemon (C.E.S. 601, S1A–5–15; S.P.I. 23028).

7. *C. macroptera* Montr. (C.E.S. 432, 3A–38–1) (fig. 9).

8. *C. medica* L.: Philippine citron (C.E.S. 757, 3A–8–5) (fig. 10); Italian citron (C.E.S. 128, 3B–2–5; S.P.I. 701); Indian citron (C.E.S. 138B, 3A–10–2).

9. **C. webberi* Wester (C.E.S. 1455, 7A–9–4; C.P.B. 10026) (fig. 11).

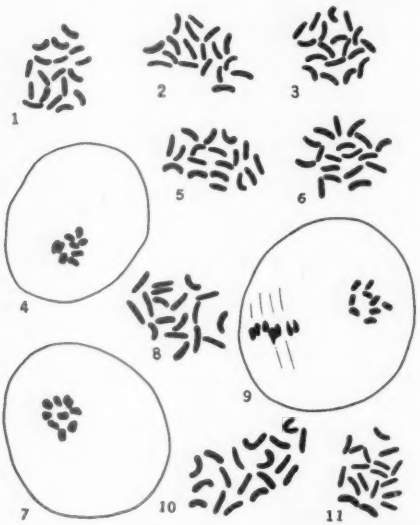
In spite of considerable differences in morphological characters and the fact that these genera and species belong to widely separated groups in the Aurantioideae, the same chromosome number (2n = 18) was found in all of them.

In table 1 the general sequence of the new classification by SWINGLE (**26**) has been followed, although many specific

[3] Citrus Experiment Station accession number and location (field-block-row-tree) in a Station orchard. C.P.B. and S.P.I. designate accession numbers of the U.S. Department of Agriculture.

[4] Throughout this paper (including the table) specific and subspecific names not considered valid by SWINGLE (**28**) are marked with an asterisk (*) *preceding* the word and so also are names of horticultural varieties not here correctly placed by species. For further explanation, see Addendum, following the Summary.

names are not acceptable to him (personal information); for instance, many of TANAKA'S species, not recognized as such by SWINGLE, are listed as they are given by NAKAMURA (18–20). In some instances horticultural variety names are also mentioned, as these may be useful to investigators of *Citrus*. To make the list as complete as possible, the writer's new determinations have also been included. In this table, tetraploid forms known to be nucellar-seedling derivatives of particular horticultural varieties have been named (by H. B. FROST) by prefixing "Tetraploid" as a part of the varietal name.



FIGS. 1–11.—Fig. 1, *Clausena lansium* (Lour). Skeels; metaphase chromosomes. 2n = 18. Fig. 2, *Atalantia citroides* Pierre ex Guill.; metaphase chromosomes. 2n = 18. Fig. 3, *Microcitrus* sp. (possibly *australasica* var. *sanguinea*); metaphase chromosomes. 2n = 18. Fig. 4, *Microcitrus* sp.; first metaphase of meiosis. n = 9. Fig. 5, *Citrus aurantifolia* Swingle (Mexican lime); metaphase chromosomes. 2n = 18. Fig. 6, *C. celebica* Koord. var. *southwickii* (Wester) Swingle; metaphase chromosomes. 2n = 18. Fig. 7, *C. ichangensis* Swingle; first metaphase of meiosis. n = 9. Fig. 8, **C. leiocarpa* Hort. var. nov. (*C. tachibana?*); metaphase chromosomes. 2n = 18. Fig. 9, *C. macroptera* Montr.; second metaphase of meiosis. n = 9. Fig. 10, *C. medica* L. (Philippine citron); metaphase chromosomes. 2n = 18. Fig. 11, **C. webberi* Wester; metaphase chromosomes. 2n = 18.

Of the thirty-three genera in this subfamily, the chromosomal constitution of only eleven is known; all these have 9 as their basic number. With regard to the occurrence of polyploidy, the following summary is presented:

TRIPLOIDS.—More than 50 triploids, or approximate triploids, have been found in the genus *Citrus*. Several triploids were hybrids between a diploid *Citrus-Fortunella* hybrid and tetraploid *Fortunella* (13, 25); about forty were hybrids between diploid *Citrus* varieties, their origin being assumed to be due to duplication of chromosomes, mainly in the nucellus, before the formation of the egg cell, perhaps through nonreduction in megasporocytes (5); twelve (LAPIN) were seedlings from open pollination; and only two, the Tahiti and Bearss limes (*C. aurantifolia* Swingle) have been recently found to be triploid varieties of economic interest. Triploids have been produced by seed parents of five *Citrus* species (if *C. paradisi* is ranked as a species).

TETRAPLOIDS.—These have so far been found in four genera (*Triphasia*, *Fortunella*, *Poncirus*, and *Citrus*); they have been found more frequently in *Citrus* (appearing in at least four species [five with *C. paradisi*], according to SWINGLE'S classification, and in numerous horticultural varieties), probably because this genus is larger and has been more extensively investigated than the others.

HIGHER POLYPLOIDS.—Beyond tetraploidy, apparently only two other forms have been detected: a hybrid pentaploid orange (BACCHI, unpublished) and a hexaploid lemon, the latter from unguarded pollination of the Ponderosa lemon (14).

TABLE 1

CHROMOSOME NUMBERS IN THE AURANTIOIDEAE

MATERIAL	NO. OF CHROMOSOMES		AUTHOR AND YEAR
	Gametic	Somatic	
Tribe 1: Clauseneae			
Subtribe 2: Clauseninae			
Clausena lansium (Lour.) Skeels	.....	18	Krug 1943
Murraya paniculata (L.) Jack	9	.....	Toxopeus(31) 1933
Tribe 2: Citreae			
Subtribe 1: Triphasiinae			
Triphasia trifolia (Burm.) P. Wilson	9	.....	Longley (15) 1925
(Tetraploid form)	18	.....	Longley, cited in Traub and Robinson (32) 1937?
Subtribe 2: Citrinae			
Severinia buxifolia (Poir.) Tenore	9	.....	Longley (15) 1925
Citropsis schweinfurthii (Engl.) Swing. & M. K.	9	.....	Longley (15) 1925
Atalantia citroides Pierre ex Guill. (possibly *A. monophylla* DC.)	.....	18	Krug 1943
**Fortunella crassifolia* Swingle	9	.....	Longley (15) 1925
F. hindsii Swingle (4n)	18	.....	Longley (15) 1925
F. hindsii (2n)	9	.....	Longley (27) 1929
F. japonica Swingle	9	.....	Longley (15) 1925
F. margarita (Lour.) Swingle	9	.....	Longley (15) 1925
Poncirus trifoliata (L.) Raf.	9	.....	Longley (15) 1925
(Tetraploid)	.....	36	Lapin (14) 1937
Microcitrus australis (Planch.) Swing.	9	.....	Longley, cited in Traub and Robinson (32) 1937?
Microcitrus sp.	.....	18	Krug 1943
M. virgata (?)*	.....	18	Krug 1943
M. australasica var. *sanguinea* (?)	.....	18	Krug 1943
Citrus aurantifolia Swingle, lime (three varieties)	9	.....	Longley (15) 1925
Lime	9	.....	Nakamura (20) 1934
Limão Seda	.....	18	Bacchi (1) 1940
Rio Claro	.....	18	Bacchi (1) 1940
*Rangpur lime, Cravo or Rosa	.....	18	Bacchi (1) 1940
*Kusai lime	.....	18	Krug 1943
Mexican lime	.....	18	Krug 1943
Palestine Sweet lime	.....	18	Krug 1943
(3n) Tahiti lime	.....	?7	Bacchi (1) 1940
Progeny of Tahiti lime	.....	19	Krug and Bacchi (11) (In press)
	.....	20	Krug and Bacchi (11) (In press)
	.....	21	Krug and Bacchi (11) (In press)
(3n) Bearss lime	.....	27	Krug and Bacchi (11) (In press)
C. aurantium L., sour orange	8	.....	Strasburger (25) 1907
(Four varieties)	9	.....	Nakamura (20) 1934
Sour orange or Agro-Sevilhana	.....	18	Bacchi (1) 1940
C. aurantium, *subspecies *bergamia* (Risso & Poiteau) (Wright & Arn. ?)	9	.....	Nakamura (20) 1934
C. celebica Koord. var. *southwickii* (Wester) Swingle	.....	18	Krug 1943
**C. deliciosa* Ten. (tetraploid)	.....	36	Lapin (14) 1937
**C. depressa* Hayata	9	.....	Nakamura (20) 1934
**C. erythrosa* Hort. ex Tanaka	9	.....	Nakamura (20) 1934
**C. genshokan* Hort. ex Tanaka	9	.....	Nakamura (20) 1934
**C. glaberima* Hort. ex Tanaka	9	.....	Nakamura (20) 1934

* *M. virgata* is a name never regularly published but tentatively assigned to a form later determined to be *M. australis*×*M. australasica* (28).

TABLE 1—*Continued*

Material	No. of chromosomes		Author and year
	Gametic	Somatic	
Tribe 2: Citreae—*Continued*			
C. grandis Osbeck, Frizzelle pummelo†	9	.....	Frost (unpublished) (1927)
(4n) Tetraploid Frizzelle pummelo	18	.....	Frost (7) 1927
Djeroek bali	9	.....	Toxopeus (31) 1933
(Seven Oriental varieties of pummelo)	9	.....	Nakamura (20) 1934
C. hystrix DC.	9	.....	Krug 1943
C. ichangensis Swingle	9	.....	Krug 1943
**C. junos* Sieb. sec. Tanaka, Yuzu and four other varieties	9	.....	Nakamura (20) 1934
**C. kinokuni* Hort. ex Tanaka	9	.....	Nakamura (20) 1934
**C. kotokan* Hayata (two varieties)	9	.....	Nakamura (20) 1934
**C. leiocarpa* Hort. (two varieties)	9	.....	Nakamura (20) 1934
Nov. var. (possibly a form of *C. tachibana* [Mak.] Tan.)	.....	18	Krug 1943
**C. limetta* Risso, Sweet lemon	9	.....	Nakamura (20) 1934
Lemon-shaped lumia	9	.....	Nakamura (20) 1934
C. limon (L.) Burm., Eureka lemon	9	.....	Longley (15) 1925
Genoa lemon	9	.....	Nakamura (20) 1934
Sicily lemon	9	.....	Nakamura (20) 1934
(Several other varieties of lemon)	9	.....	Nakamura (20) 1934
Rough lemon (see *C. verucosa*)	.....	18	Bacchi (1) 1940
Limão Doce	.....	18	Bacchi (1) 1940
Lisbon lemon	.....	18	Krug 1943
Limone Interdonato	9	.....	Ruggieri (24) 1935
Lisbon lemon	9	.....	Frost (6) 1926
(4n) Tetraploid Lisbon lemon	18	.....	Frost (4) 1925
Seedlings of Ponderosa lemon (see *C. piriformis*)	.....	18	Lapin (14) 1937
(4n, 6n) Seedlings of Ponderosa lemon	.....	36	Lapin (14) 1937
	.....	54	Lapin (14) 1937
(3n) (Four seedlings from open pollination)‡	.....	27	Lapin (14) 1937
Variegated Pink Eureka lemon§	9	.....	Frost (unpublished) (1942)
**C. limonia* Osbeck, Kusaie lime	9	.....	Nakamura (20) 1934
Rangpur lime	9	.....	Nakamura (20) 1934
Japansche citroen	9	.....	Toxopeus (31) 1933
var. *otahitensis* Tanaka, Otaheite orange	9	.....	Nakamura (20) 1934
var. *khatta* Tanaka, Khatta orange	9	.....	Nakamura (20) 1934
C. macroptera Montr.	9	.....	Krug 1943
C. medica L., citron	8	.....	Strasburger (25) 1907
	9	.....	Longley (15) 1925
var. *sarcodactylis* Swingle	9	.....	Nakamura (20) 1934
Cidra	.....	18	Bacchi (1) 1940
Philippine citron	.....	18	Krug 1943
Italian citron	.....	18	Krug 1943
Indian citron	.....	18	Krug 1943
Chinese lemon‖	9	.....	Frost (unpublished) (1942)
**C. medioglobosa* Tan.	9	.....	Nakamura (20) 1934
**C. microcarpa* Bunge, calamondin (two varieties)	9	.....	Nakamura (20) 1934
(Tetraploid calamondin)	18	.....	Nakamura (19) 1934
**C. mitis* Blanco, calamondin	9	.....	Longley (15) 1925
	.....	18	Bacchi (1) 1940
**C. natsudaidai* Hayata	9	.....	Nakamura (20) 1934

† The Frizzelle is a noncommercial polyembryonic variety with characters of both *Citrus grandis* and *C. paradisi*. See Addendum.

‡ Lapin (14) also obtained five triploid seedlings from open pollination of Shiva-mikan (species?).

§ Of unknown origin, but apparently identical, except in fruit color, with a bud-variation strain derived from the Eureka.

‖ A variety (C.E.S. 710) which seems predominantly citron in its characteristics, although the fruit is strikingly lemon-like.

TABLE 1—*Continued*

Material	No. of chromosomes		Author and year
	Gametic	Somatic	
Tribe 2: Citreae—*Continued*			
**C. nobilis* Lour. King mandarin (or orange)	9	.....	Frost (unpublished) (1927)
King mandarin	9	.....	Nakamura (20) 1934
(4n) Tetraploid King mandarin	18	.....	Frost (6) 1926
Kunembo	9	.....	Nakamura (20) 1934
**C. obovata* Rafin.	9	.....	Nakamura (20) 1934
**C. oleocarpa* Hort. ex Tanaka	9	.....	Nakamura (20) 1934
**C. paradisi* Macf. Duncan grapefruit	9	.....	Longley (15) 1925
Marsh grapefruit	9	.....	Longley (15) 1925
Marsh grapefruit (Seedy strain) (?)¶	9	.....	Frost (unpublished) (1926)
(4n) Tetraploid Marsh Seedy (?) grapefruit	18	.....	Frost (6) 1926
Imperial grapefruit	9	.....	Frost (3) 1925
(4n) Tetraploid Imperial grapefruit	18	.....	Frost (7) 1927
(4n) Tetraploid Hall grapefruit	18	.....	Frost (unpublished) (1938)
(4n) Tetraploid McCarty grapefruit	.....	36	Bacchi (unpublished)
(3n) (Seedling from open pollination)	.....	27	Lapin (14) 1937
**C. piriformis* Hassk., Ponderosa lemon (a hybrid ?)	9	.....	Nakamura (20) 1934
**C. poonensis* Hort. ex Tanaka, Ponkan	9	.....	Nakamura (20) 1934
C. reticulata Blanco, Dancy tangerine	9	.....	Longley (15) 1925
(4n) Tetraploid Dancy tangerine	18	.....	Frost (7) 1927
Willow Leaf mandarin	9	.....	Longley (15) 1925
(4n) Tetraploid Willow Leaf mandarin	18	.....	Frost (7) 1927
Unshû (=satsuma)	8	.....	Osawa (22) 1912
Satsuma orange	9	.....	Longley (15) 1925
Owari satsuma	9	.....	Nakamura (18) 1929
Wase satsuma	9	.....	Nakamura (18) 1929
Owari (?) satsuma	9	.....	Frost (unpublished) (1927)
(4n) Tetraploid Owari (?) satsuma	18	.....	Frost (7) 1927
(4n) Tetraploid satsuma	.....	36	Lapin (14) 1937
C. sinensis Osbeck, sweet orange	8	.....	Strasburger (25) 1907
Pineapple orange	9	.....	Longley (15) 1925
Valencia orange	9	.....	Longley (15) 1925
Maltese Oval orange**	9	.....	Frost (3) 1925
Paper Rind (St. Michael) orange	9	.....	Frost (3) 1925
(4n) Tetraploid Paper Rind orange	18	.....	Frost (4) 1925
Djeroek manis	9	.....	Toxopeus (31) 1933
Kinkunembo	9	.....	Nakamura (20) 1934
Lue Gim Gong orange	9	.....	Nakamura (20) 1934
Maltese Blood orange	9	.....	Nakamura (20) 1934
Parson Brown orange	9	.....	Nakamura (20) 1934
Navelencia orange	9	.....	Nakamura (20) 1934
Washington Navel orange	9	.....	Nakamura (20) 1934
(Six other varieties of orange)	9	.....	Nakamura (20) 1934
(3n) (Two seedlings from open pollination)	.....	27	Lapin (14) 1937
Laranja Doce	.....	18	Bacchi (1) 1940
Baia orange	.....	18	Bacchi (1) 1940
Imperial orange	.....	18	Bacchi (1) 1940
Ruby orange	9	.....	Frost (unpublished) (1927)
(4n) Tetraploid Ruby orange	18	.....	Frost (7) 1927
(4n) (Tetraploid orange)	.....	36	Lapin (14) 1937
Shamouti (Jaffa) orange	9	.....	Oppenheim and Frankel (21) 1929

¶ So listed, but bud-variation origin not established. For taxonomic status of grapefruit, see Addendum.

** According to Hume. This variety was formerly grown in California as Mediterranean Sweet; tree compact, semidwarf.

TABLE 1—*Continued*

Material	No. of chromosomes		Author and year
	Gametic	Somatic	
Tribe 2: Citreae—*Continued*			
**C. succosa* Hort. ex Tanaka	9	.....	Nakamura (20) 1934
**C. sunki* Hort. ex Tanaka	9	.....	Nakamura (20) 1934
**C. surcata* Hort. ex Tanaka (?)	9	.....	Nakamura (20) 1934
C. tachibana (Nak.) Tan.	9	.....	Nakamura (20) 1934
**C. tamurana* Tan.	9	.....	Nakamura (20) 1934
**C. tangerina* Hort. ex Tanaka (two varieties)	9	.....	Nakamura (20) 1934
**C. tankan* Hayata	9	.....	Nakamura (20) 1934
**C. verucosa* Hort. ex Tanaka (?), Rough lemon	9	.....	Nakamura (20) 1934
**C. webberi* Wester	.....	18	Krug 1943
**C. yatsushiro* Hort. ex Tanaka	9	.....	Nakamura (20) 1934
Subtribe 3: Balsamocitrinae			
Afraegle gabonensis (Swingle) Engler	.....	18	Krug 1943
Aeglopsis chevalieri Swingle	9	.....	Longley (15) 1925
Feronia limonia (L.) Swing.	9	.....	Toxopeus (31) 1933
Hybrids			
1. Sampson tangelo (*C. paradisi*×*C. reticulata*)	9	.....	Longley (15) 1925
2. Eustis limequat (*C. aurantifolia*×*Fortunella japonica*)	9	.....	Longley (15) 1925
3. Thomasville citrangequat (*Fortunella margarita*× [Willits citrange=*Poncirus trifoliata*×*C. sinensis*])	9	.....	Nakamura (20) 1934
4. Sacaton citraldin (C.E.S. 2619)	.....	18	Krug (n.d.)
5. (*Citrus aurantifolia*×*Fortunella japonica*)††×*Fortunella hindsii* (two hybrids)	$\frac{2}{2}^{7}$	.....	Longley (16) 1926; Traub and Robinson 32, p. 794) 1937
6. About twenty different hybrids of *Citrus*, from four seed-parent varieties of three species	$\frac{2}{2}^{7}$ (approximate)	.....	Frost (7, 8, and unpublished) 1927, 1930
7. *Citrus limon*×*C. grandis*	.....	27	Lapin (14) 1937
8. *Citrus limon*×*C. paradisi* (four hybrids)	.....	27	Lapin (14) 1937
9. Novoafon lemon×Meyer lemon	.....	28	Luss (17) 1935
10. *Citrus limon*×Meyer lemon (same as last?)	.....	28	Lapin (14) 1937
11. Fifteen other hybrids, from three seed-parent species	.....	27	Lapin (14) 1937
12. *Citrus sinensis* (Baianinha)×*C. sinensis* (Sabara)	.....	45	Bacchi (unpublished)
13. Clementine mandarin	9	.....	Lacarelle and Miedzyrzecki (12) 1937

†† Longley (16) gave *F. margarita* as the kumquat parent of this limequat, but Swingle (28) states that the limequat crossed with *F. hindsii* was the Eustis, which had the parentage here given. (See also Swingle, W. T., and Robinson, T. R., Jour. Agr. Res. 23:229–238. 1923.)

Aneuploids.—Only recently several cases of aneuploidy have been found; two (one?) hybrids were reported (14, 17) with 28 somatic chromosomes, having as male parent the diploid Meyer lemon.

Bacchi (1) found 28 chromosomes in another *Citrus* form, which has been omitted from table 1 because question has been raised as to its varietal and specific identification. In the progeny of this form, from open-pollinated flowers, eight seedlings have been found by Krug and Bacchi (unpublished) to have, respectively, 21, 21, 23, 29, 30, 33, 37, and 37 chromosomes. In spite of their odd numbers of chromosomes, some of these progeny plants seem to grow normally. In the progeny of the triploid Tahiti lime also, three aneuploids have been found by the same investigators, with 19, 20, and 21 chromosomes, respectively.

To the breeder of the genus *Citrus*,

valuable cytological data are therefore available, namely, the chromosome numbers of many of its species and also of most of its closest relatives. The fact that the basic number is the same for all of them has made it possible to make wide intergeneric crosses, as between *Citrus*, *Fortunella*, and *Poncirus;* the similarity of the chromosome complements of these three genera seems rather striking, as normal pairing has been reported to occur in such a trigeneric cross (**20**). This fact seems a challenge to the taxonomist, indicating that these three genera might perhaps rather be considered mere species of a single genus. Nothing is known regarding the evolutionary development in this subfamily. On the basis of the similarity of the genomes of *Fortunella*, *Citrus*, and *Poncirus*, NAKAMURA (**20**) points out that the evolution of species within the genera may be due mainly to the formation of new alleles. Further cytological investigations, supplemented by genetic analysis—the latter process being handicapped by the phenomenon of polyembryony—will doubtless help to elucidate the relationships in this important group of plants.

Summary

1. The chromosome number of fourteen species (twenty forms) belonging to five different genera of the subfamily Aurantioideae are reported. All determinations available in the literature on chromosome numbers made in this subfamily are tabulated. The basic chromosome number is n = 9 for all forms so far investigated. About 40 triploids, some 190 tetraploids (including 19 in *Poncirus*, 1 in *Triphasia*, and 1 wild form in *Fortunella*),[5] 1 pentaploid, 1 hexaploid, and 14 or 15 aneuploids[6] have been reported.

2. All these polyploids, except the 21 tetraploids just mentioned and several triploid hybrids between *Citrus* and *Fortunella*, belong to the genus *Citrus*. Considering the genetic similarity of the chromosome complements of several genera of the subtribe Citrinae, it is suggested that some of these might perhaps better be considered mere species of a single genus.

The writer expresses his indebtedness to the John Simon Guggenheim Memorial Foundation, which enabled him to spend several months at the University of California Citrus Experiment Station, Riverside, California, and to the director and staff of the divisions of Plant Breeding and Orchard Management of that Station for the many facilities provided.

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Addendum by Howard B. Frost
February, 1943

So far as *Citrus* forms which have been cytologically studied are adequately identified in publication by vernacular varietal names, they have usually been placed in this paper under the proper species as recognized by SWINGLE (**28**). It is impracticable, however, to rearrange in this way all the chromosome determinations that have been published under invalid specific names, partly because of

[5] The triploids doubtless were all gametic seedlings and therefore all genetically distinct, although in some cases several triploid seedlings had the same parentage. The tetraploids presumably were all of nucellar-seedling (apomictic) origin, except for one case of bud variation to tetraploidy (**9**). The number of distinct tetraploid forms (derived from different diploid forms) observed probably is considerably more than 20.

[6] This number includes a fragment trisomic (2n = 18 + 1 fragment) incidentally mentioned by BROWN (**2**); this is a citrange (*Poncirus trifoliata* × *Citrus sinensis*), variety not mentioned.

inadequacy of identification and partly because some forms evidently are interspecific or intergeneric hybrids. Further, some of these names supply a necessary group or varietal identification, such as is elsewhere furnished by vernacular names.

Some revision of this paper has recently been made at the University of California Citrus Experiment Station; and because of war conditions, MR. KRUG may not have opportunity to review the changes before publication. This revision is mainly based on SWINGLE'S (**28**) monographic treatment of the Aurantioideae, which has recently become available in printer's proof. I have also added a few *Citrus* forms, partly from previously unpublished determinations.

The names marked as invalid mainly designate groups and horticultural varieties, in some cases presumptive hybrids between *Citrus* species or even involving other genera, which do not seem to deserve specific rank. Some cases deserve mention here, as follows: *C. nobilis* Lour. was based on the King mandarin or a very similar form (**28**); the King, however, is considered by SWINGLE to be an interspecific hybrid, perhaps between *C. reticulata* and *C. sinensis*, and therefore the mandarin species becomes *C. reticulata* Blanco (the oldest valid name). In the proof of SWINGLE'S monograph, the grapefruit is distinguished as a "satellite species"; *C. paradisi*, which is closely related to the pummelo or shaddock, and the grapefruit varieties are therefore listed separately. The Rangpur and Kusaie limes are probably sour mandarins, *C. reticulata* var. *austera*, or possibly hybrids between *C. reticulata* and *C. aurantifolia*. The Meyer lemon, the Otaheite orange, the Japansche citroen (**31**), the yuzu (*C. junos*), and *C. webberi* all seem to be interspecific hybrids. The calamondin (*C. mitis* or *C. microcarpa*) is considered a *Citrus*×*Fortunella* hybrid. TANAKA'S (**30**) grouping and descriptions indicate that all the following "species" are forms of *C. reticulata: deliciosa, depressa, erythrosa, genshokan, glaberima, kinokuni, poonensis, succosa*, and *tangerina;* and also that *tankan* and *natsudaidai* are closely related to *sinensis*.

In addition to the data in table 1, NAKAMURA (**20**) found 9 chromosomes as the gametic number in nine *Citrus* forms which he did not assign to species. LAPIN (**14**) reported tetraploid seedlings, not listed in table 1, from seed parents as follows: *C. grandis, C. limon, C. natsudaidai*, and *C. paradisi;* all seed parents which gave tetraploids produced mainly diploid seedlings. In LAPIN'S cultures, as also in FROST'S, part of the tetraploids occurred among the known nucellar (apomictically produced) seedlings accompanying hybrids from interspecific crosses, and all the rest may have been nucellar; none were found among the hybrids.

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INFLUENCE OF TEMPERATURE ON PHOTOPERIODIC REACTIONS IN LEAF BLADES OF BILOXI SOYBEAN

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Introduction

The writers have studied the influence on floral initiation of various combinations of night and day temperatures applied to Biloxi soybeans for a period of 5 days during which the plants received short photoperiods (**5**). The temperature treatments were applied to the entire plant, and it was found that a night temperature of 55° F. initiated significantly fewer flower buds following the treatment with short photoperiod than those held at night temperatures of 65°, 75°, and 85° F.[3] Significant differences between lots held at a day temperature of 55° and lots held at certain higher day temperatures were also observed, but these differences were much less than those found between lots held at high and low night temperatures. The results indicated that the interaction between photoperiod and temperature was to be explained as an influence on some process or series of processes occurring during the dark period.

The photoperiodic reaction that results in flowering in Biloxi soybean plants occurs in the blade of the leaf (**2**), and the effects of this reaction are expressed in the production of flower primordia at stem growing points often located some distance from the leaf receiving the photoperiodic stimulation. The reduction in extent of floral initiation following application of low night temperatures during treatments with short photoperiod could therefore be considered as an influence of the low temperature on (*a*) the progress of photoperiodic reactions occurring in the leaf blade; (*b*) the transmission of some stimulus from the leaf blade to the terminal meristems of the stem; or (*c*) the differentiation and growth of buds from the terminal meristems. Data dealing with the last two possibilities have been presented previously (**4**). It was found in that study that floral initiation following a period of treatment with short photoperiods was much less extensive if the low temperatures were restricted either to the petioles of the leaves being subjected to short photoperiods or to the terminal meristems of plants receiving such treatment. The temperature required to reduce materially the amount of floral initiation was found to be about 37°–40°. When temperatures of 45° or higher were employed, very little inhibiting effect was observed. This inhibiting effect of temperatures of 37°–40° on floral initiation was found only when the temperatures were applied continuously, night and day, during the period of treatment with short photoperiods. This was in marked contrast to the results obtained when entire plants received a temperature of 55° during the nights only.

The results of the preceding studies (**5**, **4**) indicated indirectly that inhibition of floral initiation in Biloxi soybean plants following treatments with short photoperiods at low temperature probably resulted from an effect of the low temperature on photoperiodic reactions taking place in the leaf blades. The present paper describes apparatus designed to study this effect and presents data confirming this conclusion.

Material and methods

Each experiment involved the application of several daily 8-hour photoperiods

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[3] Temperatures throughout this paper are Fahrenheit.

and 16-hour dark periods, the latter under conditions of controlled temperature, to a single leaf blade, while all other parts of the plant were exposed to greenhouse temperatures of 70°–75° and to 16-hour or longer photoperiods. Plywood dark chambers were so designed that a single leaf blade of each of twenty plants could be readily inserted or removed without damage to the leaf (fig. 1*B*). Two such boxes, 78 inches long, 25 inches wide, and 33 inches deep, were used simultaneously. The individual leaves were placed inside the box through 3×4-inch holes cut in two sides and one end. Each hole was covered by a 5×6-inch sliding door (fig. 1*A*). The bottom of each door had a narrow slot so cut that when the door was closed over the petiole all but the top end of the slot was covered by the side of the box. The petiole occupied this upper portion so completely that almost no light could enter. Twenty such openings were provided in each box, four on one end and eight on each side. The openings extended about two-thirds of the distance along the sides of the box, leaving about one-third of the box for temperature-control equipment.

The equipment for controlling the temperature within the box consisted of a cooling coil, electric strip heaters, and a fan to circulate the air throughout the box. The temperature of the box was controlled by means of a three-wire bimetallic regulator and mercury relays, the regulator being installed in the part of the box occupied by the leaves. One side of it activated the refrigerant pump whenever the temperature exceeded the regulator setting, and the other side closed a switch in the heating circuit when the temperature fell below the setting. Water was used as the refrigerant, the temperature of which was maintained a few degrees below that desired in the box by a refrigerated constant-temperature bath. With this equipment the temperature during the dark period could be controlled within $\pm 0.5°$.

The temperature was recorded continuously by means of a soil thermograph whose bulb was suspended inside the box by the side of the thermoregulator. In addition, the temperature in the vicinity of each leaf blade was determined at 8:00 A.M. and 4:00 P.M. each day during the induction treatment, by means of thermocouples. These were installed so that when each leaf was inserted in the box the thermocouple junction was practically touching the lower side of the leaf. The temperature at each of the twenty leaves in the box was determined by means of a portable potentiometer. The continuous thermograph record in-

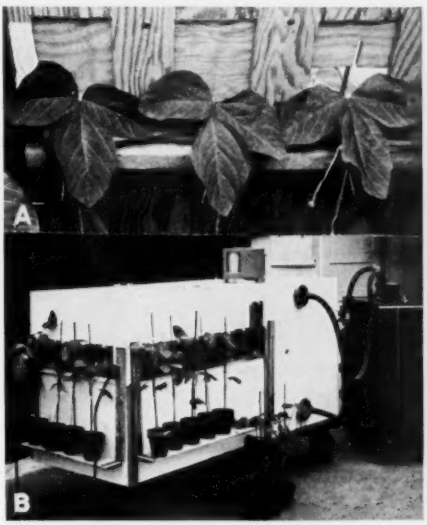

FIG. 1.—Equipment for control of temperature and photoperiod of individual leaf blades: *A*, interior of box showing leaves in position; *B*, exterior of box showing position of plants. Each plant received photoperiodic induction through one leaf inserted in box; rest of plant received long photoperiods and fluctuating greenhouse temperature.

dicated any irregularities during each experiment, while the thermocouple readings provided a measure of the uniformity of the temperature in the box at any given time.

The plants used were grown in the greenhouse during various times of the year. Both before and after the 5-day induction treatment, all lots received the natural photoperiod, followed by Mazda light of 30–50 foot-candles from sundown until midnight. During the induction treatment the selected leaf was given 8-hour photoperiods in the greenhouse from 8:00 A.M. to 4:00 P.M., whereas all the remaining leaves received light from dawn until midnight.

Five lots of twenty plants each were selected for each experiment from a population of several hundred plants. In this way the plants were closely uniform as to stage of development at the beginning of the experiment.

Two lots of twenty plants each were required for the two boxes and a third lot was used as control at greenhouse temperatures, which usually ranged from 70° to 75° during the night. The plants of this lot were placed on a greenhouse bench near the boxes, and each day during the induction period one leaf blade of each plant was covered with a double-thickness black-cloth bag from 4:00 P.M. to 8:00 A.M. One of the two remaining lots of plants was held continuously on long day and dissected at the end of the experiment; the other lot was dissected the day the experiment was started to determine the total number of nodes in the main stems and to make certain that none of the plants had initiated flower primordia prior to treatment.

Results

The experiments were conducted from December 11, 1941, to October 10, 1942, except for a few weeks during the middle of the summer when temperature control within the boxes was difficult because of excessively high outside temperatures. An attempt was made to repeat the experiments involving similar temperatures at various times throughout the year to avoid faulty conclusions that might result from seasonal variations.

The individual experiments were made with plants of different ages, and the leaf subjected to short photoperiods was also varied, depending on the size of the plant (table 1). In view of these differences in time of year, age of plant, and position of leaf, close agreement of results of individual experiments run at identical temperatures is not expected, because these various factors have been shown to influence the extent of floral initiation resulting from a given photoperiodic-induction treatment (1, 3). Nevertheless, when the data from experiments conducted at identical temperatures are averaged, the trends justify conclusions as to the effect of temperature on the photoperiodic response of a single leaf.

The efficiency of the boxes as dark chambers was tested in two preliminary experiments in which the temperature of the box was the same as that of the greenhouse. A 5-day induction period was given to one group of plants by placing the single leaves in the box and to another group by placing the single leaves in black bags. In each experiment all plants that received photoperiodic induction through the leaf placed in the box initiated flower primordia, as did also most of those that received induction with black bags. The average number of flower primordia per flower-bearing plant was 2.2 for the plants with the single leaf boxed as against 1.6 for the plants with a leaf bagged. These

results indicated that the boxes were entirely satisfactory as dark chambers and that possible light-leaks around the petioles were not causing any reduction in effectiveness of the dark period.

four experiments are not reported in table 1. In three of them none of the plants whose leaf was held at 40° during the dark period formed flower primordia. In the other experiment at 40° one plant

TABLE 1

EFFECT ON FLORAL INITIATION OF VARIOUS TEMPERATURES APPLIED TO SINGLE LEAVES OF BILOXI SOYBEANS DURING 16-HOUR DARK PERIODS OF A 5-DAY INDUCTION TREATMENT. TWENTY PLANTS USED IN EACH TREATMENT OF EVERY EXPERIMENT. EXPERIMENTS CONDUCTED DURING 1942

Experiment no.	Date of start of experiment (1942)	Age of plants (days)	Compound leaf treated	Temperature of boxes (°F.)		Total plants per lot with flower primordia			Total flower primordia per lot		
				Low	High	Low-temperature box	High-temperature box	Controls	Low-temperature box	High-temperature box	Controls
7	1/21	47	4*	45	55	3	14†	20	4	32	79
8	1/26	43	4	45	55	1	15‡	20	2	38	75
33	9/29	31	3	45	55	0	15	19	0	34	73
32	9/21	29	3	50	55	4	12	19	6	36	60
34	10/5	30	3	50	55	3	15	20	3	37	85
9	2/2	49	5	50	60	12	20	20	22	70	99
10	2/9	31	3	50	60	0	5	16	0	6	20
14	3/23	32	2	50	60	7	15	20	14	40	57
15	3/30	40	3	50	60	10	19	20	18	49	75
12	3/9	30	2	55	65	3	14	18	5	28	38
13	3/16	37	3	55	65	6	16	18	10	31	41
16	4/6	32	2	60	70	19	18	20	38	54	64
18	4/20	46	4	70	80	20	20	20	92	87	93
19	4/27	27	2	70	80	20	19	20	80	74	68
20	5/4	34	3	80	90	20	15	18	60	43	53
21	5/11	41	5	80	90	20	20	20	115	114	123
23	5/25	36	4	90	100	20	10	20	87	21	103
24	6/1	29	3	90	105	10	0	20	16	0	69
25	6/8	36	5	90	105	18	2	20	63	3	104
26	6/15	26	2	90	105	15	0	20	31	0	69
27	6/22	33	3	90	105	17	3	19	42	4	58

* Fourth compound leaf from base of plant indicated by 4, third leaf by 3, etc.
† Eighteen plants per lot instead of 20. ‡ Nineteen plants per lot.

The first four experiments were performed with only one temperature control box, which was maintained at 40°, whereas all subsequent experiments were conducted with two boxes. These first

of the twenty formed flower primordia at two nodes. The bagged controls for these experiments averaged two or more flower buds per flower-bearing plant. The long-day control plants of these four experi-

ments and of all others reported in this paper were without flower primordia at the time of dissection.

Three experiments were run in which one box was held at 45° and the other at 55°. The age of the plants varied, and the light intensity during the induction periods was different for the three experiments. All except one plant of the bagged controls in the three experiments initiated flower primordia, the average being 3.8 per flower-bearing plant. The plants that had one leaf at 55° were partially inhibited in their response, since only forty-four of the sixty formed flower buds, and these averaged 2.3 per flower-bearing plant. At 45° the stimulus resulting from short photoperiods was scarcely sufficient to induce the formation of flower buds. In one experiment three plants formed flower primordia, in another only one plant responded, and in the third none formed flower primordia.

In two other experiments the boxes were maintained at 50° and 55°. In both experiments, 50° almost completely inhibited the formation of flower primordia, since they were found in only four plants in one experiment and three plants in the other. At 55° the inhibition was not so marked as at 50°; the response at 55° was similar to that of the first three experiments.

Another series of four experiments was run with the temperature of the boxes maintained at 50° and 60°. In this series the age of the plants varied from 31 to 49 days. The older plants formed more flower primordia in all cases, but the trend toward more flower buds at higher induction temperatures was still evident. At 50° in this series the formation of flower primordia was always less than that at 60°. The small number formed on any of the plants in experiment 10 can be attributed to the fact that the plants were only 31 days old and the light intensity was very low during 2 days of the induction period. Nevertheless the trend shows that 50° definitely reduces the flower-forming stimulus. Induction at 60° also appears to result in some inhibition, because several more plants of the bagged controls held at a greenhouse temperature of 70°–75° initiated flowers than did the plants at 60°, and on the average the control plants produced a few more flower primordia.

In two experiments in which the individual leaves were held at 55° in one box and 65° in the other, the extent of floral initiation at 55° was somewhat less than in certain of the previous experiments conducted at this same temperature. In one experiment, however, the plants were very young and in the other the light intensity during the induction period was unusually low. The plants of experiment 12, although 30 days old, had made slow growth, and the second leaf was used for treatment. In experiment 13 the plants were 37 days old and the third leaf was used for treatment, but the light intensity during the 5-day induction period was very low for the first 3 days and the total for the 5-day period was about half that of the preceding experiment. The trend of the results, however, is the same as that previously reported: 55° inhibited formation more strongly than 65°, and 65° during the dark period reduced the number of flower primordia below that of the bagged controls held at greenhouse temperature.

In another experiment the boxes were maintained at 60° and 70°. The response of the plants to 60° was practically the same as previously reported. The plants whose leaves were held at 70° in the box formed nearly as many flower primordia per flower-bearing plant as did the bagged controls. This was also true for

all the experiments in which temperatures of 70° and 80° were maintained. At 90° under the conditions of these experiments formation of flower primordia was slightly inhibited in comparison with the response of the bagged controls. This inhibition was still more marked at 100°, and at 105° such formation was greatly inhibited in some experiments and completely suppressed in others.

TABLE 2

EFFECT OF TEMPERATURE APPLIED TO SINGLE LEAVES OF BILOXI SOYBEANS DURING 16-HOUR DARK PERIODS OF A 5-DAY INDUCTION TREATMENT

Temperature (° F.)	No. of experiments conducted	Total no. of plants	Total plants forming flower primordia	Percentage of plants forming flower primordia	Total flower-bearing nodes	Total flower-bearing nodes per flower-bearing plant
45	3	60	4	7	6	1.5
50	6	120	36	30	63	1.8
55	7	137	80	58	192	2.4
60	5	100	78	78	203	2.6
65	2	40	30	75	59	2.0
70	3	60	58	97	226	3.9
80	4	80	79	99	336	4.3
90	7	140	115	82	396	3.4
100	1	20	10	50	21	2.1
105	4	80	5	6	7	1.4

In one experiment not reported in the table the temperature was maintained at 110°. This temperature severely damaged the leaf receiving the 16-hour dark period, and all the treated leaves eventually died as a result of the 5-day treatment.

When the results of all the experiments at any one temperature are composited, regardless of the many other factors that differ, the trend of the effects of temperature, applied to a single leaf during the 16-hour dark period, on floral initiation becomes clear (table 2). At 45°, initiation was greatly inhibited, only 7 per cent forming flower primordia. As the temperature to which the single leaf was subjected during the long dark period was increased from 45° to 70°, the percentage of plants forming flower primordia increased. At 70°–90° there was little difference; but at temperatures higher than 90° the formation of flower primordia was definitely less.

Discussion

An earlier paper (4) suggested that the suppressing influence of low temperature on the formation of flower buds on Biloxi soybean plants that had received photoperiodic induction treatment might result from the effects of the low temperature on one or more of three processes. These processes were (*a*) those occurring in the leaf blade which in some way result in the production of a flower-inducing stimulus, (*b*) those concerned with the transmission of this stimulus from the leaf blade to the terminals of the stem where flower buds are formed, and (*c*) those resulting in the actual differentiation and development of the flower primordia at the terminal meristems. In that paper data dealing with the last two processes showed that while floral initiation could be prevented by low temperatures applied either to the petioles of leaves receiving photoperiodic induction or to the terminals of plants whose leaves were receiving such treatments, the temperatures required were relatively low (35°–45°). At 50° very little inhibiting effect was observed.

In the present investigation it was found that temperatures of considerably higher range applied to the leaf blades while they were receiving the long dark periods were effective in suppressing floral initiation. For example, a temperature of 45° applied to the leaf blade almost completely inhibited the formation of flower buds, while a similar temperature applied to the petiole or terminal was previously found to have only a moderately suppressing effect. When the leaf

blades received 55°, about half the plants initiated flower primordia; at 60° and 65° only about three-fourths of them formed flower buds. The data of this and of the preceding paper (4) therefore show that low temperature can influence floral initiation through any of the three processes mentioned, provided it is applied locally to the part concerned.

If low temperature is applied to the entire plant during the dark periods of an induction treatment, however, its inhibiting influence on floral initiation will evidently result primarily from its action on processes occurring in the leaf blade, because as the temperature is lowered from 70° these processes are the first to be suppressed. Consequently, the suppression of translocation of a flower-inducing stimulus or the inhibition of floral differentiation at the terminal meristems resulting from still lower temperatures becomes secondary, since the processes in the leaf that result in the formation of such stimulus have already become completely ineffective. Therefore the results when entire plants were subjected to low night temperature (5) are to be attributed primarily to an effect of low temperature on some chemical process or processes in the leaf blade.

In the present experiments it was found that as temperatures to which the leaf blades were exposed exceeded 90°, there was a decrease in the number of plants forming flower primordia and in the number formed per plant in response to a given photoperiodic induction treatment. Temperatures of this higher range evidently have an inhibiting influence on some process or processes in the leaf blade that are associated with the production of a flower-inducing stimulus. This reduction may come about through a direct effect on the photoperiodic reactions, or it may be the result of the effect of high temperature on secondary reactions not directly associated with flowering. The data do not indicate the reason for this response.

The upper temperature range at which reduced floral initiation became evident is not unusual in the field during the growing season, and the question arises as to whether these high temperatures may not have an inhibiting effect on floral initiation under field conditions. It seems possible that this may occasionally happen, but it is probably not common, since such temperatures seldom occur continuously throughout the night.

It is recognized that the plants were subjected to rather unusual conditions in the experiments involving high temperatures. While the entire plant with the exception of a single leaf received the temperature and humidity prevailing in the greenhouse, this one leaf was subjected to low humidity and high temperature. Although this condition favored high transpiration, the leaves in the box, even at 105°, never showed injury and appeared turgid each morning when they were removed. Nevertheless, such a high transpiration rate in the treated leaf could conceivably have caused inhibition of a flower-inducing stimulus. Such effects, however, have been observed previously when entire plants were subjected to high night temperatures (5). In those experiments the humidity was always moderately high, so it does not seem probable that any unusual conditions of transpiration could have been the causal agent in the present experiments.

Summary

1. Apparatus was devised that made possible the application of controlled temperature to individual leaves of Biloxi soybean plants during the dark periods of a photoperiodic treatment, while

the remainder of the plant received greenhouse temperatures and long photoperiods.

2. When a leaf was held at 50° F. or lower during a 5-day induction period, floral initiation was greatly inhibited. At 70°–90°, such initiation was in general equal to that of the controls held at greenhouse temperatures, but at 90° or higher the extent of the initiation again was less.

3. On the basis of the data presented in this and two preceding papers, the inhibiting effect of low temperature on floral initiation in Biloxi soybean plants appears to be the result of its effect on the photoperiodic reactions occurring in the leaf blade during the dark period, rather than through its effect on translocation of a flower-inducing stimulus from the leaf to the terminal meristems or its effect at the terminal meristems upon the differentiation and development of flower buds.

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OCCURRENCE OF ANEUPLOIDS IN LILIUM[1]

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Introduction

The genus *Lilium* is favorable material for studies in chromosome morphology, both because of its large chromosomes and because—with the exception of the triploid *L. tigrinum*—all the species reported are diploid, with 24 chromosomes. This would obviously eliminate variations due to chromosome number and thus add significance to species differences in chromosome morphology. During a study of the chromosome morphology and phylogeny of the genus *Lilium* undertaken by the writer, observation of material from approximately ninety species and varieties has revealed the presence of extra chromosomes or fragments in several forms, and a study of their behavior and effect is presented here.

Aneuploid lilies have been reported by SATO (6), who obtained plants with 24–39 chromosomes from selfs and intercrosses of *L. tigrinum*. SATO (5) also reported a *L. japonicum* with 24 chromosomes plus 2 very small fragments. These were found in somatic cells but not in meiosis, and he concluded that they were pieces of the usual 24 chromosomes broken off at constrictions. MATHER (2, 3) reported *L. henryi* with 24 plus 1 fragment and 24 plus 2 fragments. The latter was observed at meiosis, and since the fragments "sometimes paired with each other, sometimes with the long chromosomes and sometimes they were unpaired," he concluded "that they were alike, were reduplicated segments of the large chromosomes, and were not entirely homologous with any piece of any one chromosome, but were made up of a small number (about three) of pieces, each homologous with different sections of the normal complement." MATHER also examined meiosis in a *L. japonicum* with 24 chromosomes plus 2 fragments. These were described as like those of *L. henryi*, except that they were of two sizes, "the larger one appearing to consist of a piece like the smaller one, with another small section joined to it." He continued, "Since, however, SATO (1932) found the same number of fragments in *L. japonicum* as I did, it would appear that this is a clonal species." SANSOME and LA COUR (4) state that "*L. japonicum* has one or possibly two fragments." A list of chromosome numbers they obtained from root tips includes the following two, but there is no discussion of them in the text: "*formosanum*....24 (+2ff?)" and "*tigrinum splendens*....36 (+f?)." BEAL (1) found a fragment in plants of *L. willmottiae* in addition to the 12 normal bivalents. This fragment never pairs with any of the major chromosomes. It moves to one pole or the other at anaphase I, but its behavior in meiosis II has not been determined. When crosssed with *L. davidii*, a species in which BEAL found no fragment, one of the hybrids had no fragments, ten had one fragment, and one had two fragments, of which one was the size of that in *L. willmottiae* while the other was smaller.

The lilies used in the present studies were all obtained from commercial dealers in the United States and Canada, many of the species being represented by several plants from each of two or more dealers. Buds were killed in 3:1 absolute alcohol–glacial acetic acid for 24 hours and then stored in 80 per cent alcohol. Root tips were treated in 1 per cent colchicine solution 1½ hours before killing (7). Smears of both buds and roots were

[1] Scientific article no. A55, Contribution no. 1890, Maryland Agricultural Experiment Station (Botany Department).

made by the usual propiono-carmine method.

Results

Lilium pumilum (L. tenuifolium)

Chromosome counts were made in fourteen plants of *L. pumilum* from five different sources, twenty-five plants of *L. pumilum* var. Golden Gleam from four sources, and four plants of *L. pumilum* var. Red Star from a single dealer. This species is generally increased by seed, and it is known for certain that at least two of the dealers used that practice, so the plants are individuals and not vegetatively reproduced from single bulbs. In obtaining the somatic chromosome counts in this and the following species, camera-lucida drawings of each chromosome were made, the chromosomes paired, and idiograms constructed as described earlier (7). The morphology of the basic complement of 12 pairs of chromosomes was identical in all three forms.

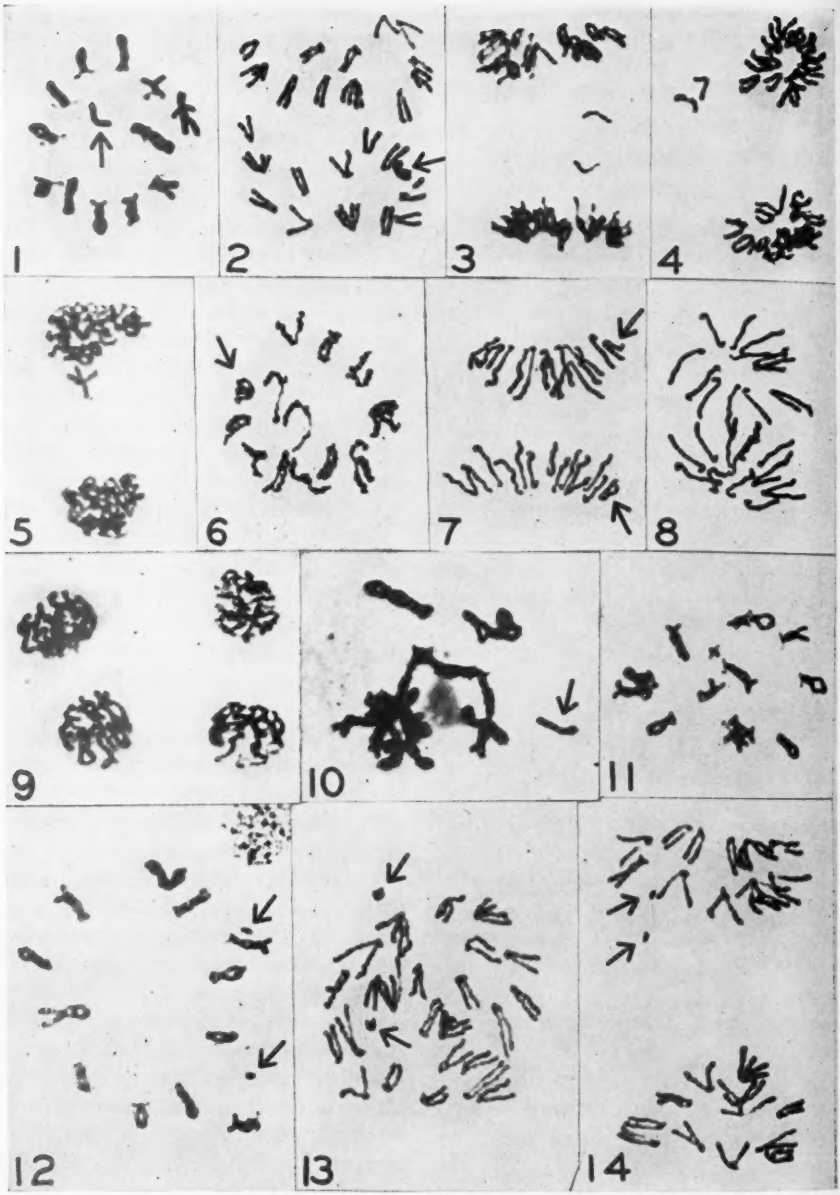
Of the fourteen plants of *L. pumilum*, nine had 24 chromosomes while the other five each had 25. The extra chromosome was the same in each case. This same chromosome was present in nine of the twenty-five plants of *L. pumilum* var. Golden Gleam but in none of the four of var. Red Star. The three forms have, like all other diploid *Lilium* species examined, two pairs of very long chromosomes with submedian kinetochores and 10 pairs of progressively shorter chromosomes with subterminal kinetochores (figs. 7, 8, 22, 23). The morphology of the extra chromosome was different from all others in that it had a median kinetochore, each arm being about the same length as the shorter arm of the chromosomes with submedian kinetochores (fig. 7).

In meiosis I, pairing was always complete in the 24-chromosome plants, but in the 25-chromosome plants there were always 12 bivalents and 1 univalent (fig. 1), the extra chromosome showing no pairing in more than 5,000 cells at diakinesis or metaphase I. In cells which were not flattened, the side view showed that it was seldom in the equatorial plate but was near one of the poles. In more than 90 per cent of the cells at anaphase I the univalent moved to a pole without splitting (fig. 2), the chromatids separated except at the kinetochore and were still tightly coiled. When it was located on the equatorial plate at metaphase I, the univalent lagged and split, the halves usually going to opposite poles (fig. 3) but occasionally to the same pole (fig. 4). At telophase I the complete univalents were sometimes at the edge of the nuclear mass (fig. 5), but then at metaphase II it was included on the plate (fig. 6) and separated normally at anaphase (fig. 7). The other nucleus of the dyad showed only the normal complement of 12 chromosomes at each pole (fig. 8). In more than 1,500 cells observed in meiosis II, no trace of split univalents—such as would result from the behavior shown in figures 3 and 4—was found. In the tetrad stage (fig. 9) not one in the thousands of cells observed had any micronuclei.

The presence of this chromosome could be detected without examining the chromosomes, as it produced a pronounced dwarfing, the 25-chromosome plants being about half the height of the 24-chromosome plants.

Three of the *L. pumilum* var. Red Star plants had 24 chromosomes, but the fourth had 26. The extra pair were alike in morphology, being very short with subterminal kinetochores.

At metaphase of meiosis I, the extra chromosomes either paired with each other (fig. 11) or were present as 2 uni-



FIGS. 1–14.—Microsporogenesis in *Lilium*. Arrows point to some extra chromosomes. Figs. 1–10, *L. pumilum* var. Golden Gleam (1, metaphase of 25-chromosome plant showing 12 bivalents and extra chromosome as univalent; 2, anaphase I, univalent moving to pole without splitting; 3, anaphase I, univalent split and halves moving toward opposite poles; 4, anaphase I, univalent split and halves moving toward same pole; 5, telophase I, complete univalent excluded from nucleus; 6, metaphase II, 13 chromosomes; 7, anaphase II, 13 chromosomes at each pole; 8, anaphase II, 12 chromosomes at each pole; 9, tetrad stage, no chromosomes excluded; 10 [*L. tsingtauense*], diakinesis showing 12 bivalents and 1 univalent). Figs. 11–14, *L. pumilum* var. Red Star (11, metaphase I, extra chromosomes paired; 12, metaphase I, extra chromosomes as univalents; 13, anaphase I, normal separation of extra chromosomes after pairing at metaphase; 14, anaphase I, both univalents moving to same pole).

valents (fig. 12), but they never paired with any of the normal complement of chromosomes. When the 2 extra chromosomes were paired, they separated normally at the first division (fig. 13) and in the second, giving 4 microspores, each with the extra chromosome. When the extra chromosomes were unpaired, they both moved without splitting, either to the same pole (fig. 14) or to different poles. In some cells both univalents split, the halves going to opposite poles (fig. 15), and in others 1 univalent split, with the halves going to opposite poles and the other univalent moving to a pole without splitting (fig. 16). The second division results in microspores with 0, 1, or 2 of the extra chromosomes, as none lag enough to be excluded from the microspore nuclei. The extra chromosomes had no marked phenotypic effect.

Lilium tsingtauense

Only one plant of this species was available. Examination of root-tip cells in the division showed 25 chromosomes. There were the usual 12 pairs plus an extra one, which had a subterminal kinetochore and was slightly shorter than the shortest of the 10 pairs with subterminal kinetochores.

Only one bud was available for study of meiosis. The microspore mother cells were in diakinesis I and metaphase I, always showing 12 bivalents and 1 univalent (fig. 10), in this respect being similar to the 25-chromosome plants in *L. pumilum* and *L. pumilum* var. Golden Gleam.

Lilium japonicum

Two plants from one dealer were examined. One had 24 chromosomes, and the other in addition had 1 very small centric fragment both in the root tips and in buds. Occasional univalents were found in the normal complement at metaphase of meiosis I, and the fragment was never paired (fig. 17). One to 2 per cent of the cells at this stage had 1–3 additional fragments of varying size (fig. 18), but these were not seen at later stages. At anaphase I the fragment sometimes lagged and divided (fig. 20), the halves going to opposite poles (fig. 19), and sometimes moved to one pole or the other without splitting (fig. 21). At anaphase II two types of distribution were found, 12+f-12 with 12+f-12 (fig. 22) and 12-12 with 12+f-12+f (figs. 23, 24). The fragments were always included in the microspore nuclei. No marked phenotypic effect of the fragment was observed.

Lilium canadense

The root tips of seventeen plants from four sources all showed 12 pairs of chromosomes. Meiosis in sixteen plants showed 12 bivalents at diakinesis and metaphase, but the other plant had in addition a small fragment which never paired with any normal complement (figs. 25, 26). This fragment divided in the first division in a few of the cells (fig. 27) but usually moved to a pole without splitting (fig. 28). Later stages were not present in the two buds from this plant. Additional roots showed no fragment present. No marked phenotypic effect of the fragment was evident.

Lilium batemanniae

The root tips of three plants from two dealers were examined. Two plants had 12 pairs of chromosomes, but one of the two from a single source had in addition a twenty-fifth chromosome possessing a subterminal kinetochore and being slightly shorter than the shortest of the 10 pairs with subterminal kinetochores. No bud material was collected. There was no marked phenotypic difference be-

FIGS. 15–28.—Microsporogenesis in *Lilium*. Arrows point to some extra chromosomes. Figs. 15, 16, *L. pumilum* var. Red Star (15, anaphase I, 2 univalents split with halves separating; 16, anaphase I, 2 univalents, 1 split and other moving to pole without splitting). Figs. 17–24, *L. japonicum* (17, metaphase I, 12 bivalents and univalent centric fragment; 18, metaphase I, 2 fragments; 19, anaphase I, fragment lagging and split, halves going to opposite poles; 20, anaphase I, univalent of normal complement and fragment lagging and splitting; 21, anaphase I, fragment univalent moving to pole without splitting; 22, anaphase II, 12-12+f distribution; 23, anaphase II, 12-12 distribution; 24, anaphase II, 12+f-12+f distribution). Figs. 25–28, *L. canadense* (25, diakinesis, 12 bivalents and fragment; 26, metaphase I, 12 bivalents and fragment; 27, anaphase I, fragment split with halves moving to opposite poles; 28, anaphase I, univalent at pole without splitting).

tween the plants with the extra chromosome and those lacking it.

Lilium willmottiae

Three plants from one source had 24 chromosomes in root tips and buds. One plant from another source carried in addition a small centric fragment both in root tips and buds. Its behavior in meiosis was similar to that of the fragment in *L. japonicum*. The fragment produced no marked phenotypic effect.

Lilium formosanum, L. tigrinum splendens, L. henryi, and others

Study of somatic chromosome morphology and of meiosis in three forms of *L. formosanum*, four collections of *L. henryi*, numerous plants of *L. tigrinum splendens*, and approximately eighty other species and varieties have revealed no fragments in addition to the complement of 24 chromosomes in the diploids and 36 in the triploid forms of *L. tigrinum*.

Discussion

The most interesting feature of the extra chromosomes and centric fragments reported here is the fact that they never pair with any of the normal complement of chromosomes, even at the earliest stages in which pairing could be seen. This complete lack of association and chiasmata formation with the basic complement of 12 pairs of chromosomes indicates that there are no blocks of homologous genes in the fragment large enough to allow any crossing-over (chiasma formation). If the extra chromosomes were duplications of blocks of material in the basic complement, although the amount of pairing would be reduced by competition with the normal homologue, nevertheless there would be some crossing-over.

There is no evidence to suggest that the species containing the extra chromosomes and fragments are hybrids. The somatic chromosomes in each case could be arranged into 12 homomorphic pairs. While a few bridges and fragments were found at meiosis in these species, indicating the presence of heterozygous inversions, these configurations were no more frequent than in any other of the "good" species of *Lilium*.

These two features, and the lack of other evidence, make it impossible to determine the origin of these chromosomes. The asexual reproduction in *Lilium* would tend to preserve the aneuploid numbers. Cytological evidence has shown that the extra chromosomes are always included in the male gametes. The fact that some of the plants of *L. pumilum* and *L. pumilum* var. Golden Gleam used in the present study carried an extra chromosome indicates that they will pass through the male and/or female gamete. Failure to find 2 extra chromosomes in one of these plants might be due to decreased viability or to failure of extra chromosome gametes to function on the male or the female side. Seeds have been obtained on selfing to test the inheritance of the extra chromosomes.

The dwarfing associated with the extra chromosome in *L. pumilum* and *L. pumilum* var. Golden Gleam indicates that it is genetically active. More extensive observation of the plants with extra chromosomes in the other species would probably reveal less marked phenotypic effects. However, no evidence was found regarding the origin or the possible phylogenetic significance of the extra chromosomes and centric fragments.

Summary

Counts of 25 and 26 chromosomes were made in plants of *L. pumilum*, *L. pumilum* var. Golden Gleam, *L. pumilum* var.

Red Star, *L. tsinglauense*, *L. japonicum*, *L. canadense*, *L. batemanniae*, and *L. willmottiae*. Approximately eighty other species and varieties showed no extra chromosomes present. The morphology of the extra chromosomes was in all cases different from that of the normal 24 chromosomes, and in meiosis they never paired with the normal complement. At least one of the extra chromosomes contains some active genic material.

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CYTOLOGY OF THE AUTOTETRAPLOID RYE[1]

T. C. CHIN

Introduction

Although in recent years colchicine has been used extensively in the induction of polyploid plants, its successful application to cereal crops has been comparatively rare.

Autotetraploid rye has been obtained by DORSEY (2) by high temperature treatment after pollination, but unfortunately no detailed cytological work was included. In 1940 Dr. H. W. LI obtained an autotetraploid rye (*Secale cereale*) by immersing the ears in an aqueous solution of colchicine during meiosis. The writer secured some of the seeds from Dr. LI, to whom he is much indebted. The smears were stained by aceto-carmine. The pollen grains and the pollen tubes were fixed by Watkins' cotton blue.

Investigation

Generally the plants of autotetraploid rye are taller, the ears are longer, and the flowers and the seeds all bigger in comparison with its diploid progenitor. The size of the cells is greater. Measurements of the stomata (fig. 1) and the pollen grains (fig. 2) are listed in table 1. It will

TABLE 1

SIZE OF CELLS OF DIPLOID AND TETRAPLOID RYE

RYE	LENGTH OF GUARD CELLS OF STOMATA	NO. OF STOMATA PER 0.01188 SQ. MM. OF LEAF			SIZE OF POLLEN GRAINS
		Upper surface	Lower surface	Ratio lower/upper	
Diploid	2.56	86.5	68.5	0.79	3.14×2.18
Tetraploid	3.26	50.0	46.5	0.93	3.72×2.98

be seen that the number of cells per unit area is comparatively less in the autotetraploid than in the diploid, owing to the greater size of the cells in the former.

[1] Contribution from the Department of Agronomy, College of Agriculture, University of Nanking.

MEIOSIS

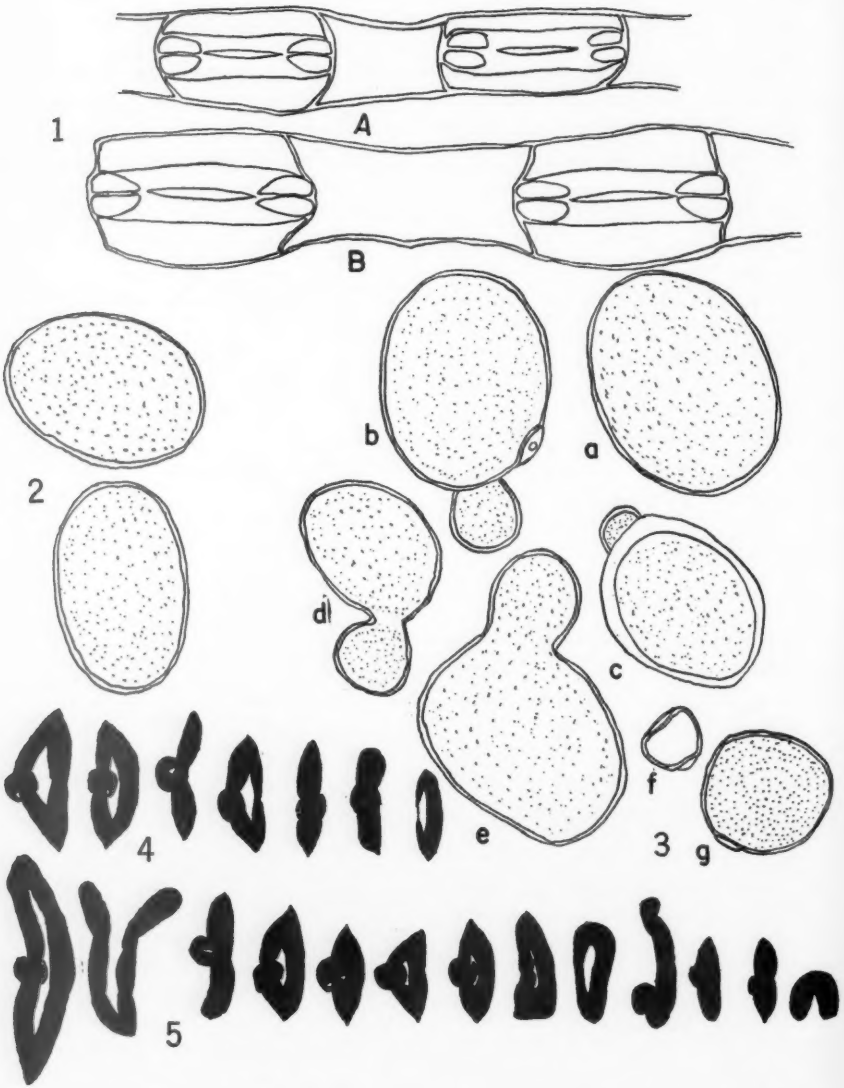
METAPHASE CONFIGURATIONS. — The diploid shows invariably 7 bivalents (fig. 4). Only rarely can two univalents be found. The meiotic division of the autotetraploid (fig. 5) shows great variations. The maximum number of multivalents is 3, and the maximum number of univalents is 5. The combinations of the configurations are given in table 2.

TABLE 2

COMBINATIONS OF CONFIGURATIONS OF TETRAPLOID RYE

Configurations	Frequency
3(IV)+8(II)	1
2(IV)+10(II)	2
2(III)+10(II)+2(I)	1
1(IV)+1(III)+8(II)+5(I)	1
1(IV)+1(III)+10(II)+1(I)	1
1(IV)+12(II)	4
1(IV)+11(II)+2(I)	2
1(III)+12(II)+1(I)	2
1(III)+11(II)+3(I)	1
14(II)	3
12(II)+4(I)	1
13(II)+2(I)	2
Total no. of cells	21

The average number of multivalents per cell is 1.05. Six out of twenty-one cells (28.6 per cent) possess no multivalents. The maximum number of chromosomes forming quadrivalents is 12/28, or 42.9 per cent. The proportion of chromosomes forming multivalents varies with organisms. Thus MÜNTZING (6) classified the autopolyploids into two main categories: (*a*) organisms wherein all the chromosomes may form multivalents, with a variable number of other configurations in different cells; (*b*) organisms wherein only a very low percentage of chromosomes form multivalents. The writer's results on the autotetraploid rye in comparison with his previous results



FIGS. 1–5.—Fig. 1*A*, stomata of diploid rye; fig. 1*B*, of tetraploid rye. Fig. 2, haploid pollen from diploid rye. Fig. 3, diploid pollen from tetraploid rye (*a*, normal pollen; *b–e*, lobed pollen; *f*, small empty pollen; *g*, small pollen). Fig. 4, configurations of first metaphase of 2n rye. Fig. 5, same of 4n rye.

on the autotetraploid barley are shown in table 3.

The difference in the frequencies of the multivalents is due to the length of the chromosomes (3, 8) and possibly also to their structure. Regarding their length, the writer found that the multivalents are almost all composed of the longer chromosomes in the 4n rye, and this, according to UPCOTT (8), is due to the possible frequency of chiasma formation. The percentage of chromosomes forming multivalents seems to be determined by the size and the possible chiasma frequency of the multivalent components and not by the total chiasma frequency of each cell. Thus in barley (*Hordeum bulbosum*), which possesses an average number of quadrivalents of 4.24 per cell, the total chiasma frequency is 25.62 in comparison with 31.10 in the autotetraploid rye, of which the average number of multivalents (quadrivalents and trivalents) is only 1.05; and even the maximum in the 4n rye does not exceed the mean number of multivalents in barley. Probably the relative lengths of the chromosome arms as well as their total lengths are the determining factors (4). No multivalents other than rings or chains were found in the 4n rye.

Tables 4 and 5 give the distribution of the chiasmata for each individual cell. The mean numbers of chiasmata per cell are 31.10 for the tetraploid and 17.00 for the diploid rye. The reduction factor in the chiasma frequency of the autotetraploid is 0.9118. This reduction is due, according to UPCOTT (9), to the greater size of cells of the polyploid plants; consequently a delay in pairing results in a reduction of chiasma frequency. The writer's results confirm this finding. The chiasma frequency, even when multivalents are absent, is always lower than twice the chiasma frequency of the diploid. The average number of chiasmata of these six cells, wherein no multivalents are found, is 32.3. The reduction factor is 0.95.

TABLE 3

DISTRIBUTION OF MULTIVALENTS IN TETRAPLOID RYE AND BARLEY

No. of multivalents per cell (4n=28)	0	1	2	3	4	5	6	7
Barley*	0	1	2	3	6	4	4	1
Chin (previous publication)	0.0	4.3	9.5	14.3	28.6	19.0	19.0	4.8
Rye†	6	9	5	1				
Chin (present paper)	28.6	42.9	23.8	4.7				

* No. of cells observed, 21; mean no. of multivalents, 4.24.
† No. of cells observed, 21; mean no. of multivalents, 1.05.

TABLE 4

DISTRIBUTION OF CHIASMATA IN AUTOTETRAPLOID RYE*

No. of chiasmata	25	26	27	28	29	30	31	32	33	34	35·····39
Frequency	1	1	0	3	3	2	2	1	1	5	1····· 1

* No. of cells observed, 21; mean no. of chiasmata per cell, 31.10.

TABLE 5

DISTRIBUTION OF CHIASMATA IN DIPLOID RYE*

No. of chiasmata	12	13	14	15	16	17	18	19	20
Frequency	1	0	2	0	1	3	2	2	2

* No. of cells observed, 13; mean no. of chiasmata per cell, 17.00.

A comparison of half chiasmata per chromosome between the diploid and the tetraploid is shown in table 6 and figure 6. The autotetraploid shows a lower number of half chiasmata for each chromosome. The distribution for the autotetraploid shows a bimodal curve, although the general trend for the two is similar, with the exception of the location of the mode.

TABLE 6

DISTRIBUTION OF HALF CHIASMATA PER CHROMOSOME IN RYE

No. of half chiasmata...	0	1	1.3	1.5	2	2.5	3	4	
Diploid.....	6	16	.....	.....	70	.....	86	4	(Mean 2.36)
	(3.3)*	(8.8)	.....	.....	(38.5)	.....	(47.3)	(2.2)	.....
Tetraploid.....	25	26	18	28	281	4	200	6	(Mean 2.10)
	(4.3)	(4.2)	(3.1)	(4.8)	(47.8)	(0.7)	(34.0)	(1.0)	

* Percentages in parentheses.

TERMINALIZATION OF CHIASMATA.—The average number of terminal chiasmata is 11.85 for the diploid and 24.76 for the autotetraploid; the coefficients of terminalization are 0.6971 and 0.7987, respectively. The higher degree of terminalization in the autotetraploid is due to the fact that the majority of the multivalents possess terminal chiasmata only.

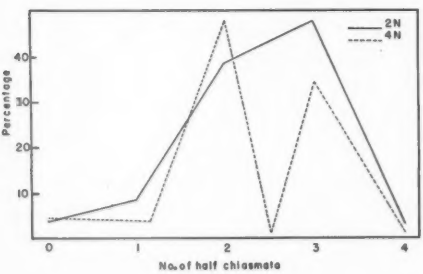


FIG. 6

INVERSION.—Bridges (figs. 7, 8) have been observed in the first anaphase; the second anaphase is normal. The total of pollen mother cells possessing a single bridge in the first division is 7.0886 per cent. The coefficient of hybridity is 0.00229. The maintenance of such a heterozygosity is due to cross pollination.

The coefficient of hybridity is not high enough to account for the reduction in the percentage of chromosomes forming multivalents, since in barley the coefficient of hybridity, amounting to 0.004 (1), is nearly 1.8 times as high as that of the autotetraploid rye. However, structural changes of this sort will eventually lead to a reduction in the chiasma frequency, and consequently the formation of multivalents is avoided (9). Then the organism will no longer be an autotetraploid but an allotetraploid.

LAGGARDS.—The laggards usually divide in the first and lag in the second division. Those which are excluded from the spindle of the first division may form microcytes (fig. 9) in the second.

GAMETE FORMATION AND STERILITY

There are two kinds of abnormal pollen grains: (*a*) abortive ones of varying size, the percentage of such grains being 2.3; (*b*) lobed grains (fig. 3). The first leads to partial sterility. The average seed setting is 23.8 per cent, ranging from 7.8 to 34.5 per cent in comparison with about 75 per cent of fertility in the diploid. Evidently the abortion of pollen grains is not the only cause of the partial sterility in the tetraploid. In addition, MÜNTZING (6) described two other causes—diplontic and haplontic sterility. The lobed pollen grains may be binucle-

ate (5, 7, 9). The main body of the grains is on the whole smaller than that of the normal ones. The size of the lobe is negatively correlated with that of the main bodies. The number of such pollen grains is 0.8 per cent. The occurrence of small pollen grains (fig. 3*f*, *g*) shows that the lobed ones owe their origin to the failure of cell-wall formation. There is no evidence to show what number of chromosomes the lobed grains may possess and even to what degree they are fertile.

Incompatibility

Cross pollination shows that only 4n × 2n (fig. 12) would result in normal seed setting, and 2n × 4n (fig. 13) is entirely incompatible. Investigation of the pollen-tube growth shows that it is simply due to the interference of the 2n growth in the 2n style (table 7).

TABLE 7

Pollen-tube growth and seed setting

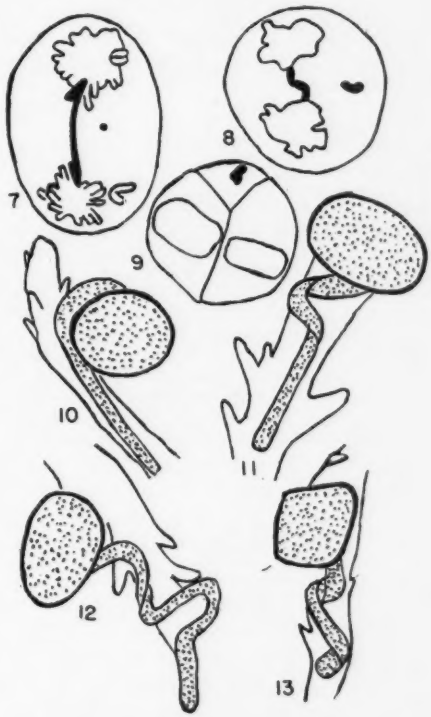
Plants	Pollen germinated (%)	Period after pollination (hours and minutes)	Length of tube growth per hour (μ)	Seed setting (%)
2n selfed....	12.4	6:30	12.24	.....
4n selfed....	8.5	5:50	12.08	33.3
4n×2n.....	10.0	5:55	11.60	38.2
2n×4n.....	2.3	6:55	3.0	0.0
2n×4n.....	5.0	22:05	*	0.0

* Total length of tube was 19.00 μ for 22 hours and 50 minutes after pollination, in comparison with 17.00 μ for 6 hours and 55 minutes.

This agrees with Watkins' (10) statement that "whereas if the tissue number be less than double that of the pollen (e.g., 2X pollen tube in 2X styles), pollen tube growth will be seriously interfered with." The difference in the behavior is due to the difference in the osmotic pressure, since the 2n pollen grains may shrink on the 2n styles.

Summary

1. The autotetraploid rye (*Secale cereale*) shows gigantism in cell size as well as in the plant as a whole.



Figs. 7–13.—Figs. 7, 8, bridge and fragment of first anaphase of 4n rye. Fig. 9, laggard and the microcyte. Fig. 10, tube of haploid pollen on diploid style, 6 hours and 30 minutes after pollination. Fig. 11, tube of diploid pollen on tetraploid style, 5 hours and 50 minutes after pollination. Fig. 12, tube of haploid pollen on tetraploid style, 5 hours and 55 minutes after pollination. Fig. 13, tube of diploid pollen on diploid style, 22 hours and 5 minutes after pollination.

2. The meiotic metaphase shows a maximum number of quadrivalents of 4, and the mean number per cell is 1.05. No multivalents were found in 28.6 per cent of the cells.

3. Chiasma frequency of the autotetraploid rye in comparison with that of the diploid is discussed.

4. Bridges of the first division have been observed. The coefficient of hybridity is 0.00229.

5. Lobed pollen grains have been found, and this is ascribed to failure of cell-wall formation. On the average, the seed setting is 23.8 per cent.

6. Cross-pollination shows that only 4n × 2n sets seed and that 2n × 4n is entirely incompatible, owing to the failure of pollen-tube growth.

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ROLE OF PYRENOIDS IN ALGAE AND OF VACUOLES IN PLASTIDS OF HIGHER PLANTS AND IN FUNGI

S. R. BOSE

Introduction

Little is known with regard to the exact function of pyrenoids in algae. According to SMITH (11), the pyrenoid synthesizes starch. When cells are grown in pure culture in total darkness but in the presence of glucose or some other simple carbohydrate, starch continues to accumulate as a series of grains around the pyrenoid. In certain of the Rhodophyceae, however, starch appears outside the plastid. In *Oedogonium*, for example, besides the pyrenoid starch there is stroma starch in the body of the sub-parallel meshes of the reticulate chloroplast. CLELAND (4) reports a close association of the pyrenoids of *Nemalion* with the formation of floridean starch, which is held to be a compound intermediate between true starch and dextrin.

In diatoms, where there is no starch sheath around the pyrenoid, SMITH (12) states that the pyrenoids possibly function as elaioplasts and may be concerned in the formation of oil. In this connection KARSTEN (7) reports that the role which pyrenoids play in starch-free diatoms remains unknown; apparently it is a constructive role in the metabolism of CO_2 assimilation, although the fatty oil in relation to pyrenoids has not definitely been observed.

Pyrenoids are probably not reserve proteins, as several are found in the youngest cells of *Oedogonium* and *Spirogyra*. Also, there are many algae (Siphonales, Myxophyceae, Charales, etc.) which lack pyrenoids but can produce starch, fat, glycogen, and sometimes even protein as reserve substances.

Pyrenoids would seem to be related to nutrition, since they become prominent during periods of abundant food supply and apparently disappear during periods of starvation. STEINECKE and ZIGENSPECK (14) consider that pyrenoids constitute receptacles of a starch-building ferment, but their experiments on changes of pyrenoids during starch production with *Spirogyra grevilleana* and *Zygnema stellinum* do not support their contention.

The present investigations were undertaken to elucidate the role of pyrenoids in algae. Observations were also made on vacuoles in fungi and in plastids of higher plants. A preliminary note on the subject has been published (3).

Observations

There is usually the synthesis of starch around the pyrenoids in green algae. In *Oedogonium* the stroma starch is deposited around the vacuoles in the body of the reticulate chloroplast and the pyrenoid starch is found around the pyrenoids, which show vacuoles at their center. In the case of starch-free diatoms, the first formation of oil takes place around the pyrenoids (fig. 1).

A faint pinkish stain is found in the pyrenoids at their center; this is true not only in cases of pyrenoid-bearing Chlorophyceae and Diatomaceae, but also in cases of *Anthoceros* and *Selaginella*, where—especially in *Anthoceros*—the "pyrenoids consist of a crowded mass of 25 to 300 disk- or spindle-shaped bodies, each of which may be transformed into a rudimentary starch grain without changing its form or position" (13). The same pinkish vacuolar stain is found in the chloroplasts of higher plants as well as of the Chlorophyceae without pyrenoids (*Vaucheria*, *Chara*, etc). In the Myxophyceae, which have no pyrenoids

and where reserve substances are usually fat and glycogen, pinkish vacuoles have been found, usually one in each cell, and oil globules are grouped around the single vacuole of each cell of the trichome.

Regarding the structure of chloroplasts in higher plants, ZIRKLE (15) states that one or more "cavities" are present in chloroplasts before starch appears in them. A faint pinkish stain is found in these cavities, and starch accumulates in the periphery of the plastid with the central vacuole. This stain is not found in old, disorganized or plasmolyzed cells of *Spirogyra*, etc. The same stain has been found (1, 2) in vacuoles of growing hyphae of diverse groups of fungi, evidently connected with the general metabolism of these fungi. From these observations the writer concludes that the vacuole probably constitutes the receptacle of enzymes which are concerned with syntheses of various food reserves found in different groups of plants.

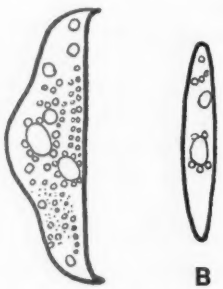


FIG. 1.—Grouping of oil drops around pyrenoids of diatoms: *A*, *Rhopalodia gibba; B, Synedra affinis* var. *fasciculata*.

DUFRENOY (6), discussing the physiological significance of the vacuome, states that the vacuoles are storage places for phenolic compounds which act as H carriers in cellular oxidation reduction and that the cytochrome oxidase has been identified in the vacuolar solution, and thus the vacuoles possess the properties of respiratory systems.

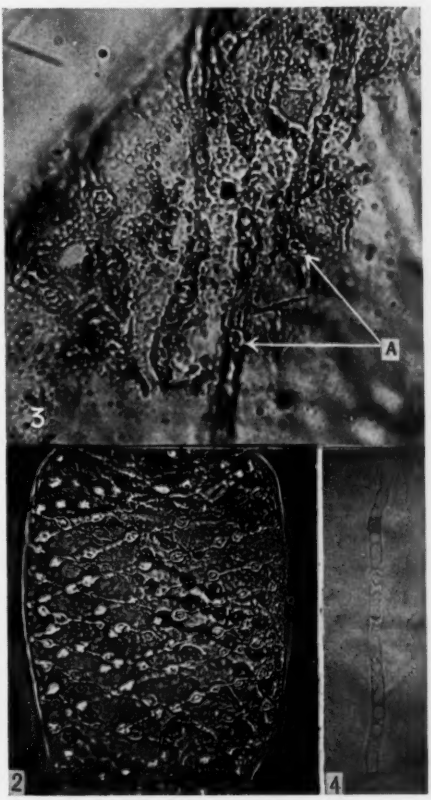
Experiments with algae

Healthy, deep-green filaments of *Spirogyra* in the vegetative stage usually show abundant starch around the pyrenoids in chromatophores (fig. 2) but no oil. PAL (9) reported a similar finding. Oil formation takes place at the conjugating stage by the gradual conversion of starch into oil. Filaments were grown in Erlenmeyer flasks in sterilized oleic acid, glycerin, and bile salt medium (designated hereafter as medium no. 1), some of the flasks being kept in darkness and others in light. Abundant oil was found in the cells of the filaments in the course of 3–5 days, some of the oil drops being actually imbedded around the pyrenoids within the starch sheath in the chromatophores (fig. 3*A*). The oil formation was greater in darkness than in light. Filaments similarly grown in sterilized distilled water as a control showed no trace of oil formation. *Spirogyra* in oleic-acid medium soon turned unhealthy and decomposed. Another fatty acid (caproic) was used in place of the oleic acid to constitute medium no. 2, and the same result was obtained. Media nos. 1 and 2 were made up in the following way: for no. 1, 1 gm. of bile salt was dissolved in 100 cc. of water and 0.5 cc. (= 130 mg.) of oleic acid and 16 drops of glycerin added, the medium being diluted four to six times with distilled water. For medium no. 2, 0.25 cc. (= 115 mg.) of caproic acid was used and the other constituents were of the same amounts. The addition of bile salt helps to make a thin emulsion, so that *Spirogyra* can continue growing for a few days. Both media were sterilized before use in an autoclave at

three atmospheres' pressure for half an hour, and the pH values of the media were about 5.8 and 6. The chemical analysis, kindly carried out by Miss ASHIMA MUKHERJEE under the direction of Dr. P. K. BOSE, confirms the utilization of oleic and caproic acids, as the amounts of both acids decreased during the process, while in two control flasks containing sterilized media nos. 1 and 2 but no filaments, the amounts of oleic and caproic acids remained practically the same.

Subsequently, glacial acetic acid and butyric acid (media nos. 3 and 4, respectively) were used. In each case two varieties (nos. 3*a* and 3*b* and nos. 4*a* and 4*b*) were prepared. In one (3*a* and 4*a*), 100 mg. of the acid was added to 200 cc. of distilled water, with 16 drops of glycerin, and in the other (3*b* and 4*b*), the glycerin was altogether omitted. All these media were sterilized, and their pH values were 5.8 after sterilization. Healthy filaments of *Spirogyra* were plunged into all the media. After 3 days in no. 3*a*, abundant oil formation was noticed in the cells of the filament, the first-formed oil drops being invariably grouped around the pyrenoids within the starch sheath, but the oil formation was much less in no. 3*b*. The same result was repeated with nos. 4*a* and 4*b*. The original starch sheath around the pyrenoids remained intact in all such cases. The chemical analyses confirm the microscopical observation on oil formation; in medium no. 4*a*, 77.5 mg. of butyric acid out of 100 mg. was used, while in the medium no. 4*b*, 67.95 mg. was used.

The estimation of free acids was carried out in each case by titrating the entire medium against the standard N/20 alkali (KOH), with phenolphthalein as an indicator. From these experiments it is concluded that oil is synthesized in the vegetative filament of *Spirogyra* growing in fatty acids and glycerin medium by the secretion of the enzyme lipase, which is probably located in the pinkish vacuoles of the pyrenoids; in the medium lacking in glycerin there was probably some secretion of glycerin from the cells for the synthesis of oil in its filaments. In all cases the first-formed oil drops were deposited around the pyrenoids. The pH value of the medium without glycerin remained the same (5.8), while



FIGS. 2–4.—Fig. 2, starch sheath around pyrenoids of normal, healthy *Spirogyra* cell. Fig. 3, grouping of oil drops (*A*) around pyrenoids within starch sheath in *Spirogyra* cell. Fig. 4, vacuoles near tip of growing hypha of *Polystictus sanguineus*.

that of the medium with glycerin was reduced to 5.5, and the filaments became unhealthy and gradually decomposed. The lowering of pH value is probably due to liberation of other organic acids by the plant during growth. This statement is further supported by the fact, as described below, that after the growth of *Spirogyra* in sterilized 4 per cent glucose medium (with pH 7 at the outset), the pH value decreased to 5.5.

To detect the presence of oil, the filaments were first treated with 0.1 per cent sodium carbonate, or in some cases with 2 per cent caustic potash for half an hour and then washed thoroughly with water to remove the adhering salts and glycerin; they were now tested by Sudan IV as well as by osmic acid. For the osmic-acid test the tannin was removed from the filaments by boiling them in water three or four times, using fresh water each time.

Spirogyra in sterilized 4 per cent glucose medium (with pH 7) in flasks maintains its green color for 8–10 days, during which period it forms abundant starch around the pyrenoids but there is no trace of oil formation. Subsequently it loses the green color and abundant oil formation is noticed around the pyrenoids within the starch sheath, which becomes perceptibly thinner and narrower, gradually decomposing, the pH value of the medium decreasing to 5.5. It has often been found that in vegetative filaments of *Spirogyra* undergoing decomposition in artificial media, as well as in yellowish green (unhealthy) filaments growing in tanks, oil drops are secondarily formed from the primarily formed starch grains situated around the pyrenoids; thus the starch sheath around the pyrenoids always becomes thinner. In all such cases the oil drops are first formed around the pyrenoids within the starch sheath as well as surrounding the sheath, apparently indicating secretion of lipase from the pyrenoids for formation of this oil.

Two species of diatoms, *Rhopalodia gibba* and *Synedra affinis* var. *fasciculata*, were grown in three sterilized media: (*a*) 2 per cent glucose, (*b*) medium no. 1, and (*c*) medium no. 1 but without glycerin. Oil developed in all three, but the amount in *b* was greater than in the other two, and the first deposit of oil in all such cases was around the pyrenoids in the form of small droplets (fig. 1).

Specimens of the Myxophyceae (*Lyngbya* sp. and *Oscillatoria* sp.) which never have any pyrenoids, when grown in 2 per cent glucose medium distinctly show by the Sudan IV test a number of oil globules grouped around the single vacuole in each cell of the trichome.

Experiments with higher plants and fungi

Short green shoots of *Selaginella* sp. were grown in sterilized medium no. 1 and in sterilized medium no. 1 but without glycerin; in both cases the leaves became discolored in 3–4 days and showed copious oil formation, the globules being mostly grouped around the pinkish vacuoles in the plastids. In 4 per cent glucose medium, oil formation took place a little later, in about 6–8 days. Normal green leaves of *Selaginella* do not contain oil; they show chloroplastids with central pinkish vacuoles and starch is usually deposited around such plastids. In green leaves of higher plants also such pinkish vacuoles inside the plastids are surrounded by a thin layer of starch.

By growing a number of fungi, *Mucor* sp., *Chaeotomium* sp., in sterilized medium no. 1 and sterilized medium no. 1 but without glycerin, it was found that

oil drops were more numerous in the medium with the glycerin. Similarly, *Chaetomium* sp., *Aspergillus oryzae*, *Polystictus sanguineus*, and *Polyporus agariceus* grown in 4 per cent glucose medium increased the amount of glycogen considerably. The chemical test by hydrolysis with hydrochloric acid and treatment with Fehling's solution confirmed the increase of glycogen. These are clear instances of synthesis of fat and glycogen by means of appropriate enzymes, which are probably located in the pinkish vacuoles of actively growing hyphae (fig. 4).

Discussion

The pinkish vacuolar stain seems very stable. It does not dissolve in alcohol, acetone, petrol-ether, ether, xylol, chloroform, iodine solution, benzol, lactic acid, acetic acid, hydrogen peroxide, pyridine, caustic potash (2 per cent) etc., although the vacuole shrinks in many cases. With Wratten filters the faint color remains visible and also under the dark-field illumination. Treated with 1 per cent antimony trichloride in chloroform solution, a slightly bluish tinge is produced in such vacuoles, surrounded by a reddish tinge, which, however, is not soluble in ether or petrol-ether, indicating that the stain is not of the nature of carotenes. Probably such vacuoles—being receptacles of enzymes—are protected by a plasma membrane (**10**). According to RANJAN, "the immunity of enzymes in living cell from the attacks of other enzymes may also be due to the membranes surrounding them. If we agree with WILLSTÄTTER that enzymes in general are adsorbed on a large colloidal carrier, forming a 'symplex,' then at the interface a membrane may be deposited, which will then act as a protective cover. Such types of enzymes will need the rupture of the cell to liberate them."

Structurally the pyrenoid consists of a central core of proteinaceous nature. According to recent views, the pyrenoid is not crystalloidal in character. CZURDA (**5**) states that the pyrenoid is a viscous mass of protein and that its interior is invariably structureless. The central portion of the pyrenoids gives the protein test with Millon's reagent. NORTHROP (**8**) has discussed crystalline enzymes in detail.

In plants with pyrenoids, syntheses of food reserves are usually found around the pinkish vacuoles of the pyrenoids. KARSTEN (**7**) states that the actual formation of oils in connection with pyrenoids is rare in starch-free diatoms. In *Rhopalodia gibba* and *Synedra affinis* var. *fasciculata*, however, the distinct grouping of all oil drops around the pyrenoids was observed (fig. 1). In plants lacking in pyrenoids, pinkish vacuoles are always found inside the plastids, and food substances are usually deposited in connection with them. In the case of fungi, which have always pinkish vacuoles at their tips (fig. 4), fat and glycogen contents can be increased by growing them in fatty acid and glucose media, respectively. The present observations and experiments indicate that vacuoles are really the receptacles of enzymes for the construction of various food substances, whether such vacuoles are located inside the pyrenoids, or plastids, or fungal hyphae.

Summary

1. In members of the Chlorophyceae with pyrenoids, starch is usually synthesized around the pyrenoids.

2. In starch-free diatoms the first-formed oil drops are grouped around the pyrenoids.

3. Healthy green filaments of *Spirogyra* in the vegetative stage usually show accumulation of starch but no oil. But by growing *Spirogyra* in fatty acids and glycerin medium, oil can be synthesized in the vegetative filaments; here also the first-formed oil drops are found around the pyrenoids within the starch sheath. Chemical analyses confirm the utilization of acids in the process.

4. In vegetative filaments of *Spirogyra* undergoing decomposition in artificial media, as well as in yellowish green (unhealthy) filaments growing in tanks, oil drops are secondarily formed from the primarily formed starch grains situated around the pyrenoids. In all such cases the oil drops are first formed around the pyrenoids within the starch sheath as well as surrounding the sheath. There must therefore have been secretion of lipase from the pyrenoids for formation of this oil.

5. A faint pinkish stain is visible under the oil immersion lens in the pyrenoids at their center. In plants lacking in pyrenoids, pinkish vacuoles are always found inside the plastids, and food substances are usually deposited in connection with them. Such vacuoles are found in growing hyphae of diverse groups of fungi, being evidently connected with their general metabolism. In many fungi, fat and glycogen contents can be increased by growing them in fatty acid and glucose media, respectively.

6. The pinkish vacuoles are considered receptacles of enzymes for syntheses of various food reserves in plants, lower as well as higher.

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EFFECTS OF GROWTH-REGULATING SUBSTANCES ON A PARTHENOCARPIC FRUIT[1]

HAROLD E. CLARK AND KENNETH R. KERNS

Introduction

In the course of experiments on the effect of several synthetic growth-regulating substances on development of the fruit of the pineapple, *Ananas comosus* (L.) Merr., a definite stimulation of the growth of the fruit has been noted following the application of relatively high concentrations of certain of them.

It has already been reported (1) that low concentrations of α-naphthaleneacetic acid applied prior to normal floral differentiation induced premature flowering of the pineapple, but high concentrations applied at the same time delayed flowering. The following data were obtained from plants treated with naphthaleneacetic acid after completion of normal floral differentiation but prior to actual blossoming (8). Some reference will also be made to results from plants treated with Fruitone, a proprietary material which includes synthetic growth regulators similar to naphthaleneacetic acid in their effects.

Experimental methods

The plants used in the experiments were of the Cayenne variety, grown in the field in the customary manner (10) in a two-row bed from a fall planting of slips. Normal floral differentiation began about 14 months after planting, as determined by sectioning and examining a few of the plants. In a uniform fall planting of this type, most of the plants start floral differentiation within an interval of about 1 week (8). From observations made in the same locality (8) of the time required for completion of the process of floral differentiation, it can be assumed that in this test the primordia of all flowers of the inflorescence had been formed within 6 weeks after the first stages—visible under the microscope—of transition from a vegetative to a floral meristem.

The experimental variables were initiated approximately 2 weeks after primordia of all flowers had been formed. At the time the treatments were applied, the young inflorescence (less than 1 inch in diameter) was still partly surrounded by leaves in the center of the plant, not having reached the stage of actual blossoming, which began about 1 month later. The plants had received adequate fertilization previously and were of average size (about 12 pounds) and quite uniform in quality of growth. Each plot included forty plants. Solutions of α-naphthaleneacetic acid (table 1) were sprayed on the surface of the leaves and inflorescence. The chemical was first dissolved in a small amount of alcohol, which was then diluted with water, and 1 liter was applied to each plot. The controls remained untreated.

Fruits were picked when ripe, each fruit being weighed to the nearest ounce. At the same time the number of fruitlets along the longest ascending spiral of the fruit (fig. 1) was counted. After the fruits had been harvested, the number of developing slips and suckers on each of the plants was recorded.

The significance of the effect of the treatments on weight of fruit and number of fruitlets was determined by analysis of variance (11). The significance of the difference between treated and control plots in numbers of slips per plant was determined by separating the plants into two categories: those with two slips

[1] Published with the approval of the Acting Director as Technical Paper no. 147 of the Pineapple Research Institute of Hawaii, University of Hawaii.

or more and those with less than two. These data were submitted to the chi-square test (11). Records of numbers of suckers were segregated and tested in the same manner.

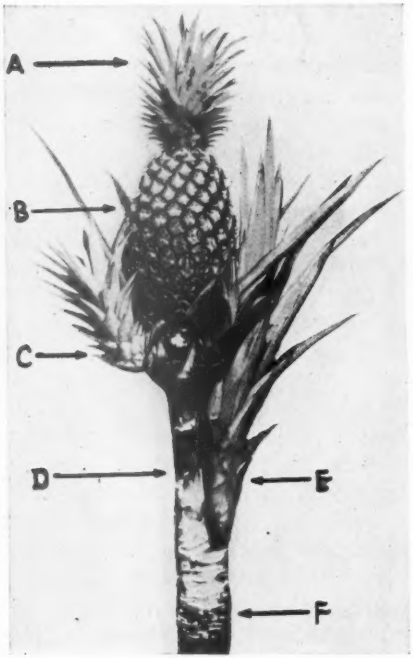

FIG. 1.—Pineapple plant at ripe-fruit stage with leaves removed from stem to show *A*, crown; *B*, fruit; *C*, slip; *D*, peduncle; *E*, sucker; and *F*, stem.

Experimental results

FRUITS

Higher concentrations of naphthaleneacetic acid definitely increased the weight of the fruits (table 1), but the lower concentrations had no significant effect in comparison with the controls. According to the calculated F values in table 1, the number of fruitlets per spiral had not been affected significantly by the treatments. This result was anticipated, since all the fruitlets presumably had been initiated and differentiation of the leaves of the crown at the apex of the fruit had been resumed when the naphthaleneacetic acid was applied. Other plots which received similar treatments considerably later (during the period of actual blossoming) gave similar results, with no real influence of treatments on the number of fruitlets but with marked increase in the size of the fruit following application of high concentrations of the acid. The highest concentration delayed ripening a week or more (fig. 2), although none of the other treatments influenced the time of ripening. A greater retardation, approximately 1 month, was obtained in other plots by application of the same high concentration prior to normal floral differentiation.

TABLE 1

INFLUENCE OF CONCENTRATION OF NAPHTHALENEACETIC ACID ON WEIGHT OF FRUIT AND NUMBER OF FRUITLETS PER SPIRAL WHEN APPLIED AFTER NORMAL FLORAL DIFFERENTIATION

	Concentration of acid (%)	Average fruit weight (lb.)	Fruitlets per spiral no.
	0.001	5.08	18.2
	.003	5.26	19.0
	.01	5.47	18.6
	0.05	5.98	18.5
	Controls	5.27	18.7
F values: Obtained		10.02	1.46
F values: Required, p=0.05		2.42	2.42
F values: Required, p=0.01		3.41	3.41
Difference necessary for significance, p=0.05		0.31	.....
Difference necessary for significance, p=0.01		0.41	.....

Further measurements of the fruits in plots referred to in table 1 were not taken, but comparable data showing some effects of a high concentration of the growth regulators on size and quality of fruit were obtained in adjacent plots.

For example, a plot treated with Fruitone at the same time as those given in table 1 can be compared with a group of four control plots. The Fruitone used was obtained in 1941, but its exact composition is not known. Other experiments with pure naphthaleneacetic acid and naphthylacetamide have shown that either stimulates growth of the pineapple fruit. The Fruitone plot received a solution calculated to provide the same amount of effective growth substance that was applied to the naphthaleneacetic-acid plot

TABLE 2

INFLUENCE OF FRUITONE ON WEIGHT AND DIMENSIONS OF FRUIT AND ON NUMBER OF FRUITLETS PER SPIRAL WHEN APPLIED AFTER NORMAL FLORAL DIFFERENTIATION

Treatment	Average fruit weight (lb.)	Fruitlets per spiral	Fruit dimensions	
			Length (cm.)	Diameter (cm.)
Fruitone....	6.54	18.7	22.4	15.3
Controls....	5.21	18.7	21.0	14.2

treated with 0.05 per cent solution. In this Fruitone plot, however, the solution was poured into the center of the plant directly on the inflorescence instead of being sprayed on the leaves. Data from Fruitone and control plots were obtained from twenty representative fruits selected from each plot (table 2).

It is apparent that the heavier, treated fruits were slightly larger in all dimensions, although the number of fruitlets was the same as in the controls, the individual fruitlets being noticeably larger in the treated fruits than in the untreated.

Within the flesh of the fruit there was no apparent anatomical effect from the growth substances. Both treated and control fruits were translucent and nonporous, that is, the tissues were well filled with juice. It should be mentioned, however, that the concentration of the juice of the treated fruits was about 2° Brix[2] lower than that of the controls (14.0° and 16.0°, respectively).

PEDUNCLES

Development of the peduncle was modified considerably by the treatments

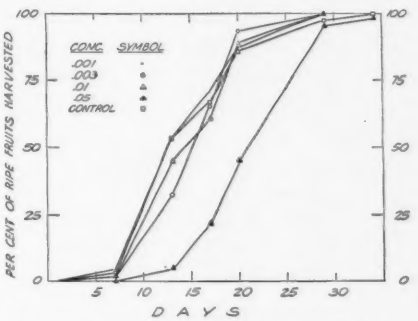

FIG. 2.—Cumulative harvest of ripe fruits from various plots according to date of ripening to show interval in days covered by harvest.

which resulted in the greatest increase in weight of fruit. Anatomical studies have not yet been completed, but in the plots receiving the highest concentration of naphthaleneacetic acid the peduncles were longer and slightly thicker than those of the controls. Furthermore, some difficulty was experienced in separating the fruits from the peduncles in these plots at the time of picking, in contrast with the ease of separation in the untreated plots or in those receiving a low concentration of the acid.

SLIPS AND SUCKERS

The treatment also had some effect on the development of axillary buds, particularly those which develop into the slips

[2] Brix hydrometer, with scale readings of percentage sugar by weight, gives some indication of sugar content of a plant sap in which sugar comprises most of the soluble solids.

(fig. 1), that is, the branches in the axils of the leaves on the peduncle. Rudimentary slip buds are present by the time the inflorescence has been formed, but most of these remain dormant and only a few develop further into axillary branches. The data in table 3 show a tendency for increase in the number of slips which developed with the lower concentrations, the result being highly significant only in the plot receiving the 0.003 per cent solution. This tendency was also noted in other plots treated somewhat later than those referred to in table 3. In contrast, plots receiving a high concentration in the center of the plant showed considerable restriction of slip development. For example, the Fruitone plot had only 0.36 slips per plant as against the 1.45 in the controls.

There was no significant influence of the treatments referred to in table 3 on the development of suckers—the axillary branches on the stem of the plant (fig. 1). In other plots high concentrations applied in the center of the plant caused some restriction in the development of the suckers, although not so marked as the inhibition of the slips in the same plots.

TABLE 3

INFLUENCE OF CONCENTRATION OF NAPHTHALENEACETIC ACID ON GROWTH OF SLIPS AND SUCKERS WHEN APPLIED AFTER NORMAL FLORAL DIFFERENTIATION

Concentration of acid (%)	Slips				Suckers			
	Average no. per plant	No. of plants with		Probability of homogeneity with controls (%)*	Average no. per plant	No. of plants with		Probability of homogeneity with controls (%)*
		One or less	Two or more			One or less	Two or more	
0.001	2.30	18	22	15	1.90	8	32	40
0.003	2.40	13	27	< 1†	2.08	7	33	25
0.01	1.35	26	14	80	1.85	10	30	80
0.05	1.42	24	16	80	1.68	17	23	15
Controls	1.45	25	15	.....	1.75	11	29	.....

* Based on chi-square test. † Significantly different from controls.

Discussion

Aitionomic parthenocarpic fruits have been produced by synthetic growth substances (**4**), including naphthaleneacetic acid (**2**) and naphthoxyacetic acid (**9, 13**). Indolebutyric acid has been used with tomatoes to increase the set of blossoms (**6, 7**), and some increase in size of fruit above that of the controls was obtained during the first few weeks of harvesting. Seedless tomato fruits produced by treatment with naphthoxyacetic acid were also reported (**5**) to be slightly larger than the controls. Although the pineapple fruit is autonomically parthenocarpic, the further stimulation of its growth by growth substances may be the result of processes similar to those occurring in fruits which are aitionomically parthenocarpic.

Although the pineapples in the plot receiving the highest concentration of naphthaleneacetic acid ripened a week later than the controls, the larger size of these treated fruits could not have been

due to a longer period of growth, because their greater size was apparent before they attained ripeness.

The pineapple fruit does not become detached from the peduncle without external force, but the difficulty in separating the fruit from the peduncle in the treated plots probably is significant in relation to known effects of naphthaleneacetic acid and naphthylacetamide upon abscission. For example, these substances have been reported to delay abscission of apples from their peduncles (3), although the concentration used was far less than that which had the greatest effect on the peduncles and fruit of pineapples.

The effect of the growth substances on the peduncles probably was not a dominant factor affecting the size of the fruit. Instead, a direct stimulation of the growth or expansion of the fruit itself by the action of the naphthaleneacetic acid was indicated.

It has been pointed out (12) that the concentration of auxin required for maximum growth varies with different organs and that higher concentrations inhibit growth. With pineapples, a relatively high concentration of naphthaleneacetic acid was apparently necessary for stimulation of growth of the fruit, and the concentration that inhibited enlargement of fruit was not reached in this experiment. A much lower concentration stimulated dormant slip buds into growth, but a high concentration inhibited the development of many slip buds.

Summary

1. Application of sprays of α-naphthaleneacetic acid to pineapple plants after normal differentiation of floral primordia had been completed influenced the growth of fruit, peduncle, and axillary branches.

2. Relatively high concentrations of the growth-regulating substances caused a marked increase in size and weight of ripe fruit, although the number of fruitlets was not influenced by any of the treatments. This increase in growth of the fruit was accompanied by the development of large peduncles, from which the fruit was separated with some difficulty.

3. The highest concentration of naphthaleneacetic acid also delayed ripening, and, particularly when applied in the center of the plant, partially inhibited growth of slips and suckers.

4. Relatively low concentrations applied at the same time caused some stimulation of the growth of dormant slip buds, resulting in a larger number of slips per plant.

The writers express their appreciation to Mr. C. A. FARDEN for assistance in statistical analysis of the data. A stimulation of their interest in growth regulators was an experiment to delay flowering by application of such substances prior to normal floral differentiation initiated by Dr. FOLKE SKOOG when at the Pineapple Research Institute.

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NUCLEOLAR STAIN AND NUCLEAL REACTION

C. S. SEMMENS

The technique introduced by SEMMENS and BHADURI (19) actually involves only three simple steps: (*a*) treatment with an alkali carbonate mordant; (*b*) staining with a dye solution; (*c*) washing out unattached dye. The method, used in conjunction with the nucleal reaction of FEULGEN and ROSSENBECK (9), as it is intended to be, involves the chemical differentiation of nucleolar material from chromatin; therefore certain essential conditions of the material, depending on previous treatments such as fixation, must be attended to if the process is to yield the required results. The nucleolar stain is designed to follow and to contrast with the Feulgen staining of chromatin; the hydrolysis necessary for the clear chromatin stain also brings about an essential change in the nucleolar material and is therefore equally necessary for the successful staining of the nucleolus by this method.

In the crystal-violet iodine technique as used for mitotic and meiotic material, the nucleolus is more or less deeply stained, and any association between chromosomes and nucleoli is difficult and usually almost impossible to determine with certainty. This is also the case with the iron-alum haematoxylin method. With the Feulgen technique the nucleoli are not stained at all, in spite of the close chemical relationship between crystal violet and Fuchsin. Here the chromosome-nucleolus relationship is again impossible to determine accurately. The Gram technique as applied to the staining of chromosomes and nucleoli does not provide a specific chromatin stain. LUDFORD (16) obtained a certain measure of success with a green dye used as a counterstain after Feulgen staining. Of course, an ordinary simple counterstain cannot serve the same purpose as a specific stain; the optical effect of dispersed green dye renders minute details of satellite or filament obscure, and there is always the physical possibility of optical resolution being hindered by dye adsorbed between filament and nucleolus. Failure to distinguish between adsorbed and attached dye has led to some rather extraordinary claims.

HILLARY'S recent use of simple counterstaining with green dye (13) has added little to the work of earlier investigators. The simple chemical difference between light green and fast green he does not mention. Moreover, the possibility that light green may form a stable or fadeless compound by actual combination with certain types of material (as it does with the nucleolus in my experience) is seldom taken into account.

Whether the nucleolar staining method of SEMMENS and BHADURI (3, 4) actually satisfies the technician's demand for a definite and specific stain for nucleolin, to go with the only definite and specific stain for chromatin (technique of FEULGEN and ROSSENBECK), is a matter for the test of time and expert consideration. Verbatim repetition of unsound criticism (7) is not authoritative opinion.

The original paper of SEMMENS and BHADURI (19) concerned a method of differentiating nucleoli from chromosomes. The reference to the advisability of avoiding acetic acid in the fixative had therefore nothing to do with the chromatin staining, although HILLARY (12) seems to think that it had. The Feulgen technique is an established method; the paper referred to by HILLARY was concerned with the nucleolus. The wording in that paper was not so obscure as to make it appear that the caution about acetic acid referred to a possible effect on the Feulgen staining, especially as this

action of acetic acid had already been indicated by the work of earlier investigators (16, 1, etc.).

Acetic acid was found to have a definite action on the nucleolus, as a result of which the nucleolin, after acid hydrolysis and subsequent treatment with alkali carbonate, would not retain the green dye if the material were subjected to adequate washing. The change that actually takes place in the constitution of nucleolin as a result of the action of acetic acid has a chemical significance which will be discussed in another article. HILLARY'S reference to the action of acetic acid relates to the chromatin staining by the Feulgen technique. The remarks about acetic acid in the paper on nucleolar staining by SEMMENS and BHADURI concern the nucleolar staining and should not be disregarded. To stain nucleoli specifically by this method, acetic acid should be avoided in the fixative and chromic acid used, either in the fixative itself or as a subsequent mordanting bath. Although the technique is extremely simple, the essential chemical conditions for the final reaction are necessarily slightly involved. One is the action of metabisulphate as a nucleolar mordant for acid green dyes which has been previously referred to by SEMMENS (17), and HILLARY'S (13) staining appears to be a good sample of this action. In my experience, however, the method is not applicable: in many cases the stain is fugitive, and it is much less distinctive than the effect after separate carbonate mordanting.

JOHANSEN (15) has stated that the Fuchsin stain in the chromosomes is "often completely removed," presumably by the alcoholic carbonate used as a mordant for the nucleolus. This difficulty has never arisen in our laboratory, and it is hard to understand from the chemical point of view why it should occur if the technique is strictly followed. It has been assumed (17–20) that the effect of alkalies on Fuchsin staining would be generally known, and no warning was therefore thought necessary about the action of the mordant.

In the mordant solution, and in the alcohols used for washing out surplus mordant, preparations normally appear almost colorless and may probably have been regarded as spoilt, since little chromatin stain is apparent under the microscope. The usual reddish magenta color of the chromatin reappears, however, in the subsequent treatment of the material, if the correct period of hydrolysis has been given (30 minutes at 60° C. for chromic-fixed plant material, 20 minutes at 40° C. for animal material). Preparations should not be discarded simply because they appear colorless in alkaline solution. The Fuchsin dye is chemically linked to the chromatin by the Feulgen and Rossenberg technique, and although internal rearrangement of linkages in the complex molecule thus formed may take place under the influence of mild alkali, leading to a temporary leuco condition, the chromogen is readily re-established when the alkali is completely washed out in the rinsing alcohols and the prevailing pH sufficiently lowered by the subsequent treatments. In the experience of a number of cytologists the final appearance of the chromatin is rather more brilliant after the nucleolar staining than with the simple Feulgen technique alone, the chromatin specificity of the Feulgen stain being in no way impaired. It is possible that loss of color after the mordant is because the solutions for subsequent treatment were somewhat on the alkaline side, and those who have difficulty possibly adhere to the time and tempera-

ture of hydrolysis—4 minutes at 60° C.—tentatively suggested by FEULGEN in the original paper.

Light green was chosen as a stain for the nucleolus in the nucleolar staining method (**19**) on account of its acid nature and its value as a contrast to magenta. As pointed out by SEMMENS (**17, 18**), fast green works equally well but gives a bluish tinge, which, though it does not appear to be satisfactory to the writer, may have definite advantage for those whose sensitivity to green is less keen than to blue. A number of other dyes of similar chemical constitution to light green will also give equally good results if applied according to the technique.

The dye solution itself is a critical factor in the technique, and if attempts to stain the nucleolus specifically with ordinary light green solutions lead to failure, such failure must not be credited to the S. & B. nucleolar technique. A trace of aniline in the solution is essential to the dye linkage, but the presence of much nitrobenzene (the most usual impurity with aniline oil) is distinctly inimical and will lead to disappointing results. If the method, as recently published (**2, 20**), is carefully adhered to, good preparations can be obtained with little trouble. As with all new techniques, however, patience is necessary at first.

The nucleolar staining method actually introduces only a single stage in addition to the ordinary procedure for double staining with Feulgen technique and a counterstain, as used by LUDFORD (**16**), HILLARY (**13**), and others. This additional stage, however, is a very important one—the application of a mordant for a specific secondary staining purpose subsequent to the attachment of a separate dye in a primary locus. It is the use of a mordant for the purpose of introducing a second stain specific for a given subsidiary component after the application of a primary stain specific for a major component that constitutes the new feature. This mordant treatment makes all the difference between a generally dispersed counterstain, with its characteristic optical effects, and a definitely located or specific stain.

In previous publications dealing with the nucleolar technique the terms "mordant" and "differentiating solution" have been used for convenient description. The main features of the nucleolar staining method are here compared with those of the nucleal reaction:

Nucleal reaction	Nucleolin reaction
Certain preliminary restrictions on nature of fixatives.	Certain preliminary restrictions on nature of fixatives.
New treatment for material; acid hydrolysis at 60° C. in N/HCl after fixation.	New treatment of fixed material; mild alkali hydrolysis subsequent to acid hydrolysis and treatment with sulphite solution.
Special dye solution for specific staining.	Special dye solution for specific staining.
Unattached dye removed with special acid solution.	Unattached dye removed with special alkali solution.
Time of hydrolysis and temperature tentative and subject to variation to suit material.	Strength of mordant and length of immersion tentative and subject to adaptation to suit material.

Consideration of the elementary chemistry of dye constitution will make it apparent that:

Nucleal reaction	Nucleolin reaction
The substitution of crystal violet (18), malachite green, pararosaniline, methyl green, etc., for basic Fuchsin could not be regarded as constituting new techniques.	The use of fast green, Guinea green, aniline blue, etc., or similar acid dyes in place of light green or mordant treatment after dye (instead of before it) could not be considered as new techniques.

SHEFFIELD (21), employing the S. & B. technique for comparative purposes in connection with very exacting cytochemical investigations on plant viruses, reports favorably on its use. CASPERSSON and SCHULTZ (5) have suggested a chemical explanation for the action of the mordant and for the reaction between dye and nucleoli. The possibility of adequate mordanting, for the green dye to be retained by the nucleoli of certain materials, simply by treatment with potassium metabisulphite solution after staining in Fuchsin was pointed out some time ago (17) and has been borne out more recently by HILLARY (13) and DELAY (8).

If the material is not adequately washed in the so-called differentiating solution after treatment with green dye, the dye will adhere rather tenaciously in such loci as lend themselves to adsorption (for example, the surface of chromosomes and certain small characteristic cytoplasmic inclusions). Such adsorbed dye may lead to questionable interpretations (14); thorough and careful washing, however, should remove all dye held simply in physical relation.

BHADURI (2) has provided evidence of the value of the combined nucleal and nucleolin techniques, as well as of the inadequacy of many of the earlier staining methods in the matter of differentiating and rendering visible the finer details of nuclear organization. Already the volume of literature on the chemical aspects of cytology is reaching considerable proportions, and a number of cytologists (6, 10, 11, 22, and others), some of whom formerly concerned themselves mainly with the morphological and mechanical aspects of chromosome behavior, are now showing awareness of the significance of this modern trend.

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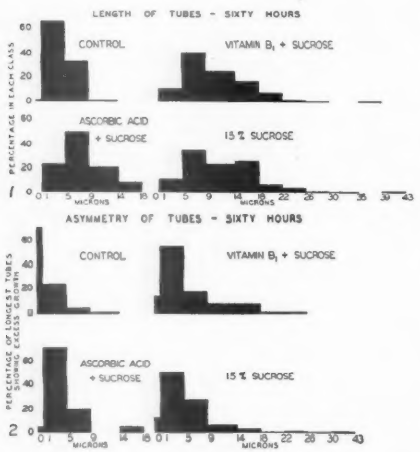
Attempts to germinate the pollen of the common dandelion are of interest, since there appears to be no record of successful germination of composite pollen *in vitro* (3), and since natural germination has not been recorded (2). This species is apogamous, the embryo developing from a diploid egg; the typical pollen is likewise diploid, but variations ranging from normal reduction to formation of supernumerary grains by chromatin extrusion have been observed (1).

Taraxacum officinale Weber., or *T. vulgare* Lam. Schrk., was tested for germination under a



FIGS. 1, 2.—Fig. 1, percentage of tubes of various lengths after 60 hours under different nutrients. Fig. 2, excess growth of longest tubes in each lot after 60 hours, shown in percentages.

variety of conditions. Ordinary hanging-drop preparations of the usual media failed to give results, but definite, intact, tubular extrusion through all three germ pores was obtained when the pollen was floated on 15 per cent sucrose with coverglass arranged to admit air. The preparations were kept in a small moist chamber at 24°–26°C. As compared with the distilled-water control, in which the tubular extrusions ranged 0–13 μ in length (fig. 1), those of the pollen treated with 15 per cent sucrose were 0–42 μ. The addition of growth-promoting substances (thiamin, ascorbic acid, 3-indoleacetic acid, 3-indolebutyric acid, and 3-indolepropionic acid) had no effect on the maximum length attained. However, the addition of thiamin to the sucrose increased the number of grains showing tubes from 26 to 67 per cent. Addition of ascorbic acid decreased "germination" from 26 to 22 per cent. Other growth-promoting substances, such as takadiastase, yeast extract, and naphthaleneacetic acid, when added to the sucrose produced no tubes larger than those in distilled water. Effects resembling those of ascorbic acid were produced by 3-indoleacetic acid and also by 3-indolebutyric and 3-indolepropionic, but to a less marked degree. The addition of boron had no effect on the action of sucrose.

These tests indicated the tendency of one tube to surpass the others in length. In distilled water only 25 per cent of the tubes surpassed either of the other two by as much as 1–4 μ, and none beyond 5 μ (fig. 2). However, 15 per cent sucrose, both alone and in combination with thiamin or ascorbic acid, had marked effect in producing a single tube or extrusion longer than the two remaining.

The graphs represent conditions after the lapse of 60 hours. In general, the great majority of grains reacting were 30–34 μ in diameter, and presumably diploids formed in dyads. There was no fixed relation between pollen size and magnitude of response, as measured by length of tubes or ascendency of one over the others in length. If tube lengths more than twice the pollen diameter or tubes showing nuclei (3) are accepted as criteria of germination, it cannot be maintained that true germination was secured. The operation of simple osmotic or colloidal swelling is not definitely excluded as an explanation. These seem unlikely, however, in view of the rather frequent predominance of a single tube, the concentration of nutrient used, and the failure to obtain similar phenomena with variations of pH. The rather frequent predominance in length of a single tube, its intact character, and the definite effect of certain nutrient and growth-promoting materials all suggest that true growth was initiated.—ELAINE J. KATZ, *Oberlin College, Oberlin, Ohio.*

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CURRENT LITERATURE

Practical Plant Anatomy. By ADRIANCE S. FOSTER. New York: D. Van Nostrand Co., 1942. Pp. 155. $2.50.

This compact volume, indicative of present interest and development in the field of plant anatomy, is more nearly a scholarly manual than a comprehensive treatise. The author, recognizing the number and merit of extensive textbooks covering this area, presents an instruction program in more concise form. He aims with a minimum of discussion and direction to guide the student, both in his reading and in his laboratory study. The essence of theory, with copious references, is combined with the study of living and macerated material as well as prepared slides.

For each topic an introduction provides necessary descriptions and definitions, as well as critical discussion of facts, theories, and interpretations, against a classic background. The laboratory directions which follow are somewhat elastic and are applicable either to a general course or to more extended study in plant anatomy. The use of diagrams and notes is recommended in partial substitution for the necessarily tedious drawings of complex structures or tissues. Use of living material is encouraged where possible, and histological technique in general is left to the special manuals. Each chapter concludes with a list of references.

The subject is approached through consideration of the cell wall, meristems, cell types, etc., with appreciation of the functional point of view rather than strict classification of tissues by their method of development. There is repeated recognition of progressive dedifferentiation and its various applications. The omission of all illustrations may lead to some difference of opinion. Obviously this permits the inclusion of more material per given number of pages and favors compactness. The plan should promote independent or inductive study rather than identification of material in the laboratory. Since the author stresses fundamentals and gives less attention to variations in given cells and tissues, the program will probably facilitate achievement under the guidance of a competent instructor.—ROBERT B. WYLIE.

Aquatic Phycomycetes (Exclusive of the Saprolegniaceae and Pythium). By FREDERICK K. SPARROW, JR. Ann Arbor: University of Michigan Press, 1943. Pp. xix+785. Figs. 637. $5.00.

For some time there has been increasing need for a book in English comparable with MINDEN'S *Kryptogamenflora der Mark Brandenburg.* This need has now been met by SPARROW.

The introduction covers such matters as phylogeny, occurrence, etc.; and precise information on how to collect, purify, and culture the saprophytic forms is assembled for the first time. In discussing phylogeny, SPARROW agrees with SCHERFFEL in believing that the Phycomycetes have arisen from the monads and developed independently along uniflagellate and biflagellate lines. The introduction ends with a key to the orders, of which there are eight of good standing, with a ninth of doubtful position. These orders are dealt with fully, except for the omission of the genera *Synchytrium, Physoderma,* and *Urophlyctis* (all parasites on land plants) from the Chytridiales, the family Saprolegniaceae from the Saprolegniales, and the non-aquatic members and *Pythium* from the Peronosporales. Apparently diagnoses of *Olpidium brassicae* and related species are omitted because of their parasitism on land plants.

The treatment of each order consists of a well-documented, terse introductory part in which the morphological and physiological researches are reviewed. This is followed by the systematic part, with keys and technical diagnoses, totaling 8 orders, 21 families, 108 genera, 475 species, and 10 varieties. Twelve of these genera and some 40 of the species were originally described by SPARROW. Among these diagnoses are many which were first published in various foreign languages. These have been translated, either literally or in modified form, and brought together in a way to make them usable by all English-speaking students.

Over half the book (369 pages) is devoted to the Chytridiales, after removing the Plasmodiophorales and Hyphochytriaceae from this order. This allocation of space is justified, for while all the other orders treated have been monographed previously in English, this is the first comprehensive treatment of the Chytridiales to appear in English and is far and away the most complete in any language. A list of substrata, a bibliography of about 720 titles, and an index complete the book.

The 637 figures, many of which are after SPARROW, are well chosen and beautifully reproduced. Binding and typography are excellent. The frontispiece shows photographs of DANGEARD, ZOPF, PETERSEN, and CORNU. This volume will doubtless remain the outstanding work in this field for a long time; even when superseded, it will still rank as a classic.—J. N. COUCH.

Timbers of the New World. By SAMUEL J. RECORD and ROBERT W. HESS. New Haven, Connecticut: Yale University Press, 1943. Pp. xv+640. Pls. 58. Maps 8. $10.00.

This volume is the culmination of more than 25 years of intensive investigation and is based on the Yale collection of woods, including about 20,000 Latin American specimens. Encyclopedic in nature, this work will be useful primarily to wood technologists, tropical botanists, and foresters, and of interest to explorers, importers, botanists, and laymen in general. It supplies much essential and interesting information in a logical and simple manner—much of it never before assembled in such an accessible form.

Thirty pages and several plates are devoted to gymnospermous trees, from *Araucaria* to *Taxodium*. Illustrations range from the Alerce (*Fitzroya*) of Patagonia to white pine in Utah and Douglas fir in Washington.

Angiospermous families, numbering 148, are also arranged alphabetically. Each family is briefly characterized, with emphasis on general distribution and gross features of stem, leaf, flower, and fruit. The American genera are listed and their economic importance noted. Specific qualities of the wood, such as color, specific gravity, grain characteristics, and cellular details, are summarized. The number of genera and species is indicated for each family.

More than 1000 genera are treated individually in most cases, with separate paragraphs for prominent species. When known, all essential qualities of the wood are given for each species, including density, compressibility, bending tolerances, elasticity, fiber stress, texture, durability, etc.

Frequent citations to recent botanical and technological articles are included. Naturally the discussion of such well-known genera as *Quercus* and *Acer* is limited to the more important commercial species, while the discussion of other genera summarizes all extant information. A comprehensive list of common names for each species for each nation or region is given.

Seventy-five photomicrographs of representative woods, a bibliography for each major geographic subdivision of the western hemisphere, lists of families classified according to special properties, and uses of bark, leaf, or timber, together with a complete index, enhance the value of this book.—PAUL D. VOTH.

Rio Mayo Plants: A Study of the Flora and Vegetation of the Valley of the Rio Mayo, Sonora. By HOWARD SCOTT GENTRY. Carnegie Institution of Washington Publication 527, 1942. Pp. vii+328. Pls. 29. Figs. 6. Maps 2.

Improved transportation facilities to some of the many parts of northwestern Mexico still unexplored by botanists, and the thorough work of the author, extending over 7 years, have resulted in this valuable floristic study. Rio Mayo originates in the high mountains of Chihuahua and flows southwestward through the State of Sonora, reaching the Gulf of Mexico during rainy seasons. Geography, climate, information concerning travel facilities, an ecological discussion of the thorn, short-tree, oak, and pine forests, a record of secondary succession on abandoned fields, and lists of cultivated plants with Mexican and Indian vernacular names precede the floristic list which constitutes the greater part of this volume. Collections number 3200, representing 1276 species and varieties of which 90 have been described as new. The interpenetration of subtropical and desert plants in this region is emphasized by this study. Photographs and typography are excellent.—PAUL D. VOTH.

Common Edible Mushrooms. By CLYDE M. CHRISTENSEN. Minneapolis: University of Minnesota Press, 1943. Figs. 62. $2.50.

Those who are interested in mushrooms from a culinary standpoint will find this volume helpful in determining species which may be eaten with safety. Tests for identification are reasonably direct and simple. The abundant clear-cut illustrations are purposeful.—E. J. KRAUS.

GENERAL INDEX

A

Acrophorus, development of sorus and prothallium 437
Algae, role of pyrenoids 633
Allison, F. E., Biochemical nitrogen fixation studies 63
Anatomical studies of roots of pine 443
Anatomy, and histology of Eureka lemon 288; of date fruit 426
Apple fruit, morphology and histology 3
Apples, respiration in storage 178
Arabogalactan, hydrolysis by Aspergillus 329
Arnon, D. I., Composition of the tomato plant influenced by nutrient supply 576
Aspergillus, hydrolysis of arabogalactan by 329
Atwood, Alice C., "Geographical guide to floras of the world" 189
Aurantioideae, chromosome numbers 602
Auxins, comparative activity of synthetic 281
Avery, G. S., Comparative activity of synthetic auxins and derivatives 281
Avocado blossom buds, differentiation 323

B

Baldwin, H. I., "Forest tree seed" 367
Bates, J. C., Varietal differences in anatomy of wheat grain 490
Bausor, S. C., Effects of growth substances on reserve starch 115
Beal, J. M., Cytological studies in relation to the classification of the genus Calochortus. III 553
Bean, affected by platinum chloride 161; mineral nutrition and light 133
Beeson, K. C., Effects of micro-nutrient deficiencies on growth and vitamin content of the tomato 495
Berger, J., Comparative activity of synthetic auxins and derivatives 281
Blair, Winifred M., Device for measuring entry of water into roots 152
Blake, S. F., "Geographical guide to floras of the world" 189
Bonner, James, Effects of application of thiamin to Cosmos 475
Borthwick, H. A., Influence of temperature on photoperiodic reactions in leaf blades of Biloxi soybean 612
Bose, S. R., Role of pyrenoids in algae 633
Buchholz, J. T., Obituary sketch of C. J. Chamberlain 369

C

Calochortus, classification of the genus in relation to cytology 553
Cave. M. S., Development of macrogametophyte of Miersia 185
Chamberlain, C. J., Obituary sketch 1, 369
Chandler, W. H., "Deciduous orchards" 188
Chemical changes accompanying development of peach 348
Chin, T. C., Cytology of the autotetraploid rye 627
Chlorophyll-protein complex 449
Chlorophylls *A* and *B*, effects of solvent upon absorption spectra 515
Chloroplast substance of spinach leaves 122
Chromosomal association, during meiosis in Dactylis 171; in clones of Dactylis 541
Chromosome numbers in Citrus 602
Citrus, chromosome numbers 602
Clark, H. E., Effects of growth-regulating substances on pineapple 639
Colchicine, effect on Colchicum and Chlamydomonas 50
Comar, C. L., Chloroplast substance of spinach leaves 122
Convolvulus, development of root system 417
Cooper, G. O., Microsporogenesis and development of seed in Lobelia cardinalis 72
Cornman, Ivor, Susceptibility of Colchicum and Chlamydomonas to colchicine 50
Cosmos, effects of thiamin 475
Couch, J. N., book review 651
Cranberry Glades, vegetation and substrate 371
Cryptomitrium tenerum 264
Cucurbita, anatomy 394
Cytological studies of Calochortus 553

D

Dactylis, chromosomal association during meiosis 171; variance and covariance of chromosomal association 541
Darlington, H. C., Vegetation and substrate of Cranberry Glades 371
Date fruit, anatomy 426
Defoliation of tomato plant 486
Differentiation of avocado blossom buds 323
Dissociations of cellular proteins and incipient drought 480
Dorf, Erling, "Upper Cretaceous floras of Rocky Mountain region" 494
Dormancy of spores of Phycomyces affected by growth substances 224

E

Eaton, S. V., Influence of sulphur on metabolism of black mustard 306; Volatile sulphur content of black mustard plants 82

Echinocystis, anatomy 394
Effects of, growth-regulating substances on shoot development of roses during common storage 26; growth substances on reserve starch 115; photoperiod on microsporogenesis in soybean 99; light quality and mineral nutrition on bean 133; micronutrient deficiencies on vitamin content of tomato 495; nutrient-solution concentration on growth of Marchantia 591; platinum chloride on bean and tomato 161; solvent upon absorption spectra of chlorophyll 515; sulphur deficiency on metabolism of mustard 306; thiamin on Cosmos 475; vaporous naphthoxyacetic acid on development of tomato 362; vitamins on germination of orchids 455
Ellis, G. H., Effects of micro-nutrient deficiencies on growth and vitamin content of the tomato 495

F

Fishman, M. M., Chlorophyll-protein complex 449
Floral stimulus in Xanthium, translocation 409
Ford, E. S., Anatomy and histology of the Eureka lemon 288
Foster, A. S., "Practical plant anatomy" 651
Frazier, J. C., Nature and rate of development of root systems of Convolvulus 417
Fruiting of tomato plant influenced by nutrient supply 576

G

Gaseous emanations from tomato fruit and defoliation 486
Gentry, H. S., "Rio Mayo plants" 652
Growth substances, effects of 26, 50, 115, 161, 224, 243, 281, 338, 362, 455, 475, 486, 639, 650

H

Hamner, C. L., Effects of platinum chloride on bean and tomato 161; Treatment of seeds with synthetic growth-regulating substances 338
Harris, D. G., Effects of solvent upon absorption spectra of chlorophyll 515
Haupt, A. W., Studies in Californian Hepaticae 264
Hayward, H. E., Device for measuring entry of water into roots 152
Hepaticae, Californian 264
Hill, Helen D., Variations in chromosomal association and behavior during meiosis in Dactylis 171
Histological responses of Vicia faba to indoleacetic acid 243
Hoagland, D. R., Composition of the tomato plant influenced by nutrient supply 576
Hoover, S. R., Biochemical nitrogen fixation studies 63

I

Iris fulva, cell size in developing ovaries 90

K

Karling, J. S., "Plasmodiophorales" 366
Katz, Elaine J., Pollen-tube development in Taraxacum 650
Kavanagh, Virgene and Frederick, Growth substances and dormancy of spores of Phycomyces 224
Kerns, K. R., Effects of growth-regulating substances on pineapple 639
Kraus, E. J., book reviews 188, 366, 367
Krug, C. A., Chromosome numbers in the subfamily Aurantioideae 602

L

Land, W. J. G., Obituary sketch 1
Lee, F. A., Chemical changes accompanying development of peach 348
Legume nodules, excised 63
Legumes, species relationships shown by electric mobility curves 449
Lemon, anatomy and histology 288
Lilium, occurrence of aneuploids 620
Lloyd, F. E., "The carnivorous plants" 494
Lobelia, microsporogenesis in 72
Long, E. M., Developmental anatomy of the fruit of the date 426
Lowenhaupt, Benjamin, Nutritional effects of boron on growth of sunflower 316
Lutman, B. F., book review 366
Lyon, C. B., Effects of micro-nutrient deficiencies on growth and vitamin content of the tomato 495

M

Marchantia, effects of nutrient concentration 591
Marth, P. C., Effects of growth-regulating substances on shoot development of roses during common storage 26
Measuring entry of water into roots 152
Meiosis in autotetraploid Secale 563
Mentha, spindle abnormalities 273
Meyer, S. L., Physiological studies on mosses. IV 128
Microsporogenesis and development of seed in Lobelia cardinalis 72
Miersia, development of macrogametophyte 185
Minor elements, review of Third Supplement 189
Minor, F. W., Biochemical nitrogen fixation studies 63
Mitchell, J. W., Effects of vaporous naphthoxyacetic acid on development of tomato 362
Morrow, Dorothy, Cell size in developing ovaries of Iris fulva 90
Mosses, physiological studies 128
Moyer, L. S., Chlorophyll-protein complex 449
Mustard plants, sulphur content 82, 306
Myers, W. M., Analysis of variance and covariance of chromosomal association in clones of Dactylis 541; Variations in chromosomal association and behavior during meiosis in Dactylis 171

N

Nelson, Ray, Spindle abnormalities in Mentha 273
Nielsen, C. S., Effects of photoperiod on microsporogenesis in Biloxi soybean 99
Nightingale, G. T., Potassium and phosphate nutrition in pineapple 191
Nitrogen fixation studies 63
Noggle, G. R., Effects of vitamins on germination of orchids 455
Northen, H. T., Relationship of dissociation of cellular proteins by drought to physiological processes 480
Nucleolar stain and nucleal reaction 639
Nutritional effects of boron on growth of sunflower 316
Nutrition, mineral, of bean 133; of pineapple 191

O

Olmsted, C. E., book reviews 188, 189
O'Mara, J. G., Meiosis in autotetraploid Secale 563
Orchids, germination and growth 455
Owens, H. S., Optimal conditions for the hydrolysis of arabogalactan by Aspergillus niger 329
Ownbey, Marion, Cytological studies in relation to the classification of the genus Calochortus. III 553

P

Palser, Barbara F., Histological responses of Vicia faba to indoleacetic acid 243
Parker, M. W., Influence of temperature on photoperiodic reactions in leaf blades of Biloxi soybean 612
Parthenocarpic fruit, affected by growth substances 639
Patterson, Sister Mary T., Cytology of apogamy in Polysticum 107
Peach, chemical changes accompanying development 348
Peas, transplantation experiments 460
Petunia pollen, X-ray treatment 528
Photoperiod, effects on soybean 99, 612
Phycomyces, dormancy of spores affected by growth substances 224
Pine, anatomy of root 443
Pineapple, effects of growth substances 639; potassium and phosphate nutrition 191
Platinum chloride, effects on bean and tomato 161
Polysticum, cytology of apogamy 107
Preston, R. J., Anatomical studies of roots of pine 443

R

Raspberry canes, histological study 356
Ratajak, E. J., Optimal conditions for the hydrolysis of arabogalactan by Aspergillus niger 329
Record, S. J., "Timbers of the New World" 651
Reece, P. C., Differentiation of avocado blossom buds 323
Reed, H. S., "A short history of the plant sciences" 188
Regeneration in Physcomitrium 128
Respiration of apples in storage 178
Reynolds, Mary E., Development of the node in Ricinus 167
Ricinus, development of the node 167
Rick, C. M., Cyto-genetic consequences of X-ray treatment of pollen in Petunia 528
Riley, H. P., Cell size in developing ovaries of Iris fulva 90
Robbins, W. J., Growth substances and dormancy of spores of Phycomyces 224
Robbins, W. W., "Weed control" 367
Rohrbaugh, L. M., Effects of light quality on growth and mineral nutrition of bean 133
Root, measuring water uptake 152; system of Convolvulus 417
Rosenberg, H. R., "Chemistry and physiology of the vitamins" 188
Roses, effects of growth substances during storage 26
Rye, cytology of the autotetraploid 563, 627

S

Salisbury, E. J., "Reproductive capacity of plants" 366
Schopf, J. M., book review 494
Scott, Flora M., Anatomy of Echinocystis and Cucurbita 394
Secale, meiosis in autotetraploid 563, 627
Seeds of radish and oats treated with growth substances 338
Semmens, C. S., Nucleolar stain and nucleal reaction 639
Shalucha, B., Comparative activity of synthetic auxins and derivatives 281
Shull, C. A., book reviews 188, 189
Simonds, A. O., Histological study of raspberry canes 356
Skaling, P. E., Device for measuring entry of water into roots 152
Skok, John, Defoliation of tomato plant as response to gaseous emanations 486
Smock, R. M., Influence of controlled-atmosphere storage on respiration of apples 178
Soybean, effects of photoperiod 99; influence of temperature on photoperiodic reactions 612
Sparrow, F. K., "Aquatic Phycomycetes" 651
Spinach leaves, chloroplast substance 122
Spindle abnormalities in Mentha 273
Starch reserve, effects of growth substances 115
Stewart, R. N., Occurrence of aneuploids in Lilium 620
Stewart, W. S., Treatment of seeds with synthetic growth-regulating substances 338
Storage, apples and respiration 178; roses affected by growth substances 26
Sulphur content of mustard plants 82
Sunflower, nutritional effects of boron on growth 316

Susceptibility of Colchicum and Chlamydomonas to colchicine 50
Swanson, C. P., Spindle abnormalities in Mentha 273

T

Taraxacum, pollen-tube development 650
Tehon, L. R., "Fieldbook of native Illinois shrubs" 494
Temperature, influence on photoperiodic reactions of soybeans 612
Thomson, Betty F., Development of sorus and prothallium of Acrophorus 437
Tomato, affected by growth substance 362; affected by micro-nutrient deficiencies 495; affected by platinum chloride 161; composition influenced by nutrient supply 576; defoliation of plant affected by gaseous emanations 486
Translocation of floral stimulus in Xanthium 409
Transplantation experiments in peas 460
Tukey, H. B., Chemical changes accompanying development of peach 348; Gross morphology and histology of developing fruit of the apple 3

V

Van Dersal, W. R., "Ornamental American shrubs" 367
Varietal differences in anatomy of wheat grain 490
Vegetation and substrate of Cranberry Glades, West Virginia 371
Vicia faba, histological responses to indoleacetic acid 243
Vitamin content of tomato 362, 495
Vitamins, effect on growth of orchids 455
Voth, P. D., book reviews 494, 651, 652; Effects of nutrient-solution concentration on growth of Marchantia 591

W

Water entry into roots, device for measuring 152
Went, F W., Transplantation experiments in peas 460
Wheat grain, varietal differences in anatomy 490
Whitehead, Muriel R., Effects of vaporous naphthoxyacetic acid on development of tomato 362
Winter injury of raspberry canes 356
Withrow, Alice P. and R. B., Translocation of the floral stimulus in Xanthium 409
Wylie, R. B., book reviews 494, 651
Wynd, F. L., Effects of vitamins on germination of orchids 455

X

Xanthium, translocation of floral stimulus 409
X-ray treatment of pollen in Petunia 528

Y

Young, J. O., Gross morphology and histology of developing fruit of the apple 3

Z

Zscheile, F. P., Effects of solvent upon absorption spectra of chlorophyll 515